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# Physiological Zoölogy

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## STUDIES ON THE NUTRITION AND REPRODUCTION OF PARAMECIUM[1]

(Four figures)


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IN THE last few years the methods of controlling the food in protozoan cultures have been more and more refined. Hargitt and Fray (1917) were the first workers who attempted to raise ciliates on pure cultures of bacteria. They isolated thirty species of bacteria from their hay-infusion cultures and identified eight of them. Using pure cultures of these bacteria in hay infusions, they found that only one of them, *Bacillus subtilis*, would support growth of *Paramecium aurelia* in isolation cultures. These cultures were carried for 12 days. Hargitt and Fray were unable to raise *Paramecium* on pure cultures of *Pseudomonas fluorescens;* but Oehler (1920) found that he could obtain good mass cultures of *Paramecium caudatum*, *P. aurelia*, and *P. putrinum* on pure cultures of *Pseudomonas fluorescens*.

Phillips (1922) used pure cultures of *Bacterium coli*, *Bacillus cereus*, *Proteus vulgaris*, and nine unidentified species of bacteria in hay infusions for the growth of *Paramecium aurelia*. She found that only one of these, an unidentified *Streptothrix*, when used alone would support the growth of *Paramecium*. Philpott (1928) found that *Paramecium caudatum*, *P. aurelia*, and *P. calkinsi* could be


[1] The author wishes to acknowledge his indebtedness to Dr. W. C. Allee for his interest and advice throughout the course of the work.

made immune to the toxic effects of the pathogenic bacterium *Bacillus pyrocyaneus*. He was able to raise *Paramecium* for 100 days on a pure culture of this pathogenic form in hay infusions. He was not able to obtain growth of *Paramecium* using *Bacillus enteritidis*. Chejfec (1929) studied the bacterial requirements of *Paramecium* for the maintenance of life and found that normal division rate of a single animal would not occur in cultures with concentrations of *Bacterium coli* less than 24,000 per cubic millimeter. Glazer and Coria (1930) report that they were able to raise *Paramecium caudatum* on pure cultures of one species of unidentified bacteria. Their attempts to obtain growth with several other kinds failed. Raffel (1930) was able to grow *Paramecium aurelia* for a considerable period using a single species of bacterium, *Bacillus candicans*, and a single species of alga, *Stichococcus bacillaris*, as food. Losina-Losinsky (1931), in studying the feeding reactions of *Paramecium caudatum*, found that bacteria form the chief food substance of *Paramecium* and that some bacteria are taken in in greater numbers than others. *Paramecium* was found to react quite positively to suspensions of *Bacillus subtilis*, less positively to suspensions of *Bacillus fluorescens liquefaciens*, and very slightly to suspensions of *Bacterium coli communae*. The most recent work of this nature on *Paramecium* is that of Phelps (1934). He found that *P. aurelia* would grow well for 50 days on pure cultures of *Erythrobacillus prodigiosus* in a particulate lettuce medium. Reviews of similar studies on other Protozoa may be found in the papers of Luck, Sheets, and Thomas (1931), Johnson (1933), and Phelps (1934).

Myers (1927) studied the course of populations in cultures of *Paramecium caudatum*, using different volumes of hay infusion and different numbers of initial seedings. He found that all cultures raised to a maximum population and then declined. When he used 0.2 cc. of medium, the cultures with one and two animals at the start reached the highest maximum, with the two-animal cultures reaching the maximum first. The four- and eight-animal cultures in the same volume did not reach as high maximum populations. When he used 0.4 cc. of hay infusion, the one-, two-, and four-animal cultures reached about the same maximum populations, with the four-animal culture reaching the maximum first and the

one-animal culture last. The eight-animal culture had a lower maximum population.

The purpose of this investigation has been (1) to study the ability of *Paramecium caudatum* to grow on suspensions of pure cultures of different bacteria in a balanced physiological salt solution and (2) to investigate the course of populations of *Paramecium* in different densities of bacteria.

## MATERIALS AND METHODS

The animals used in these experiments were taken from a mass culture of *Paramecium caudatum* which has been kept in this laboratory for the last seven years. A modification of the method described by Parpart (1928) was used in sterilizing the ciliates. In the paired experiments sister-organisms from a pure line were used in each experiment.

The balanced physiological medium which Barker and Taylor (1931) used in raising *Colpoda cucullus* on suspensions of *Pseudomonas fluorescens* and which Johnson (1933) used in growing *Oxytricha fallax* on suspensions of *Pseudomonas fluorescens* was used in these experiments. The concentrated medium was diluted to the approximate concentration of pond water. One cubic centimeter of M/20 $NaH_2PO_4$ was added to each 30 cc. of this diluted medium for buffering, and the hydrogen-ion concentration was adjusted with small amounts of M/20 NaOH to pH 6.8–7.0, which has been found to be well within the optimal pH range for *Paramecium.*

Cultures of the following kinds of bacteria, which were grown on agar plates, were used: *Escherichia coli*, *Aërobacter aërogenes*, *Bacillus subtilis*, *Pseudomonas fluorescens*, *Serratia marcescens*, *Staphylococcus aureus*, and one unidentified yeast. The bacteria were removed from the plates with a platinum loop and suspended in the medium. The bacteria, taken from a loop which measured 2.5 mm. in diameter and placed in 10 cc. of the medium, formed a suspension which is referred to later as "$x$" concentration. A $5x$ concentration was produced by adding five times the amount of bacteria.

Ground-glass protozoan isolation dishes were used in all of the experiments. Evaporation in the cultures was maintained at a minimum by keeping the dishes in moist chambers. Capillary

pipettes were used in transferring the animals. All glassware used in each experiment was sterilized before it was used. Counts were made with the low power of a binocular microscope. The temperature during these experiments varied from 23° C. to 28° C. Two drops of the medium were equal to 0.1 cc., and 5 drops to 0.25 cc.

## EXPERIMENTS WITH DIFFERENT KINDS OF BACTERIA

Eight series of experiments were run using pure suspensions of the six kinds of bacteria, of the yeast, and of the six kinds of bacteria and the yeast mixed together in 2 drops of medium. These experiments were carried for a 10-day period with the temperature controlled at 27° C. Counts were made at 48-hour intervals. Six individuals were isolated singly into each kind of suspension. After each count six individuals were isolated into fresh suspensions of the different kinds. If there were not six individuals left at the time of any count in any series, a sufficient number of paramecia were taken from the stock culture to make up the difference. Table I gives the results of these experiments.

TABLE I

SHOWING GROWTH OF *Paramecium* ON SUSPENSIONS OF DIFFERENT BACTERIA

| | 2-Day Periods | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | | 2 | | 3 | | 4 | | 5 | |
| | S* | C† | S | C | S | C | S | C | S | C |
| Yeast | 6 | 12 | 6 | 22 | 6 | 1 | 6 | 24 | 6 | 5 |
| *Escherichia coli* | 6 | 21 | 6 | 9 | 6 | 8 | 6 | 9 | 6 | 1 |
| *Aërobacter aërogenes* | 6 | 14 | 6 | 11 | 6 | 0 | 6 | 13 | 6 | 4 |
| *Pseudomonas fluorescens* | 6 | 12 | 6 | 13 | 6 | 3 | 6 | 18 | 6 | 6 |
| *Staphylococcus aureus* | 6 | 19 | 6 | 10 | 6 | 3 | 6 | 22 | 6 | 9 |
| *Serratia marcescens* | 6 | 24 | 6 | 6 | 6 | 3 | 6 | 11 | 6 | 12 |
| *Bacillus subtilis* | 6 | 52 | 6 | 60 | 6 | 40 | 6 | 52 | 6 | 48 |
| Mixed | 6 | 23 | 6 | 0 | 6 | 18 | 6 | 12 | 6 | 2 |

* Starting number. † Number counted.

The 10-day period is divided into five 2-day periods. Six animals were started in each culture, and the numbers found at the end of 48 hours are listed under C. An examination of these results shows that only one of the eight types of suspensions, those of *Bacillus*

*subtilis*, supported good growth of *Paramecium*. There was some division in all of the cultures, but in no case did the division rate equal that found in the suspensions of *Bacillus subtilis*. Also, there was not a single other type of suspension which supported continuous growth for the 10-day period.

SHOWING GROWTH OF *Paramecium* IN SUSPENSION OF *Bacillus subtilis*

A short experiment was conducted isolating five animals singly into 2 drops of *x* concentration of *Bacillus subtilis*, with the temperature controlled at 27° C. Counts were made at the end of 24 hours, and five more cultures were started in fresh material. The animals isolated on succeeding days were taken from the preceding cultures. This was done for 6 days, with the following average fission rates: 1.3, 1.2, 1.5, 1.0, 1.0, and 1.4.

TABLE II

SHOWING GROWTH OF *Paramecium* IN 5 DROPS OF *Bacillus subtilis* SUSPENSION

| Series | Average Fission Rate | Series | Average Fission Rate | Series | Average Fission Rate |
|---|---|---|---|---|---|
| 1 | 2.6 | 7 | 2.2 | 13 | 2.2 |
| 2 | 1.8 | 8 | 1.1 | 14 | 3.4 |
| 3 | 1.3 | 9 | 1.3 | 15 | 2.3 |
| 4 | 1.3 | 10 | 2.0 | 16 | 1.6 |
| 5 | 1.5 | 11 | 1.5 | 17 | 1.2 |
| 6 | 1.9 | 12 | 1.9 | Av. | 1.8 |

Table II gives the results of a 51-day experiment using 5 drops of medium of the same concentration. The cultures in this experiment were carried for 3 days in each case, out of necessity. Each figure represents the average fission rate for the first day of each series of cultures. Here, as in the case mentioned above, each figure represents the average division rate for 1 day of five organisms isolated singly. The temperature in this experiment varied from 25° C. to 28° C.

Obviously, there has been no gradual lowering of the fission rate over this period. These results indicate that *Paramecium caudatum* may be cultured successfully in suspensions of a pure culture of *Bacillus subtilis* in a non-nutritive balanced physiological medium for a 51-day period, at least.

GROWTH OF *Paramecium* IN DIFFERENT DENSITIES OF *Bacillus subtilis*

In the first part of this work nine series of experiments were conducted, each lasting for 3 days. In each series 5 drops of $5x$ concentration of bacteria and 5 drops of $x$ concentration were used. In each concentration five animals were started together and five others

TABLE III

USING $5x$ AND $x$ CONCENTRATIONS OF *Bacillus subtilis*

| Cultures | $5x$ Concentration | | | $x$ Concentration | | |
|---|---|---|---|---|---|---|
| | 1 Day | 2 Days | 3 Days | 1 Day | 2 Days | 3 Days |
| 1. Groups | 1.0 | 2.9 | 3.35 | 1.2 | 1.6 | 1.6 |
| Singles | 1.0 | 2.4 | 4.03 | 1.5 | 2.3 | 2.4 |
| 2. Groups | 1.4 | 3.25 | 4.38 | 1.8 | 3.0 | 3.2 |
| Singles | 1.2 | 3.0 | 5.15 | 2.0 | 3.45 | 3.73 |
| 3. Groups | 1.5 | 3.15 | 3.45 | 1.5 | 2.1 | 2.25 |
| Singles | 1.1 | 2.1 | 3.65 | 1.3 | 2.35 | 3.03 |
| 4. Groups | 1.6 | 3.3 | 3.65 | 1.7 | 2.05 | 2.3 |
| Singles | 1.2 | 3.0 | 3.9 | 1.8 | 2.65 | 2.8 |
| 5. Groups | 1.0 | 2.85 | 3.58 | 1.7 | 2.2 | 2.55 |
| Singles | 1.2 | 2.55 | 3.85 | 1.5 | 2.6 | 3.05 |
| 6. Groups | 1.8 | 3.4 | 4.44 | 2.0 | 2.4 | 2.55 |
| Singles | 1.1 | 2.8 | 5.0 | 1.9 | 3.38 | 3.8 |
| 7. Groups | 1.6 | 3.08 | 3.75 | 1.6 | 2.4 | 2.5 |
| Singles | 0.9 | 2.65 | 4.85 | 1.8 | 3.28 | 3.53 |
| 8. Groups | 1.8 | 3.45 | 3.8 | 2.25 | 2.65 | 2.65 |
| Singles | 2.3 | 3.4 | 3.85 | 2.7 | 3.1 | 3.15 |
| 9. Groups | 1.3 | 2.85 | 4.0 | 2.05 | 2.75 | 2.8 |
| Singles | 1.3 | 2.55 | 4.08 | 2.2 | 3.05 | 3.1 |

were isolated singly. Counts were made at 24-hour intervals. The temperature in these experiments was that of the room and varied about the same as reported for the experiments of Table II.

Table III gives the results of these experiments. Each figure represents the average fission rate of five animals since the beginning of the experiment. These results show a number of things. At the end of one day in the culture with $5x$ concentration there is no significant difference between the groups and the singles. By the end

of the second day in the $5x$ concentrations the groups are reproducing faster than the singles. The statistical significance here is 0.0026. At the end of the third day the singles have gone ahead of the groups. In this case the statistical significance is 0.0052.

In the $x$ concentrations there is no significant difference between the groups and singles at the end of the first day. But the difference between them during the second and third days is very marked. The singles consistently have a higher rate of reproduction than the groups.

An examination of the groups in the two concentrations shows that the groups in the $x$ concentrations reproduce slightly faster during the first day than the groups in the $5x$ concentrations. The

TABLE IV

5-DAY CULTURES IN $5x$ AND $x$ CONCENTRATIONS

| Concentration | Days | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| $5x$ groups | 1.2 | 3.08 | 3.87 | 4.04 | 3.97 |
| $5x$ singles | 1.05 | 2.7 | 4.58 | 4.72 | 5.1 |
| $x$ groups | 1.5 | 2.3 | 2.4 | 2.4 | 2.23 |
| $x$ singles | 1.75 | 2.87 | 3.06 | 3.14 | 3.17 |

statistical significance is 0.0108. However, after the first day the groups in the $5x$ concentrations have a consistently higher rate of division.

The singles in the $x$ concentrations reproduce significantly faster during the first day than the singles in the $5x$ concentrations. There is no significant difference between them during the second day, but at the end of the third day the singles in the $5x$ concentration have reproduced markedly faster than those in the $x$ concentration.

With this start on the course of populations of *Paramecium* in different densities of bacteria, it seemed advisable to carry similar experiments for a longer time. Table IV gives the results of similar experiments carried over 5-day periods. This table represents the average of two sets of experiments over a 10-day period. Each figure is the average fission rate of ten individuals. When the results of

the first 3 days in this series are compared with the results given in Table III, the same general relationships are found to exist. During the 5-day period the group culture apparently reached their maximum population, those in the $5x$ concentrations by the end of the fourth day, and those in the $x$ concentrations by the end of the third day. The singles in both concentrations continued to increase during the 5-day period. The graphs in Figures 1 and 2 show the trends of these populations.

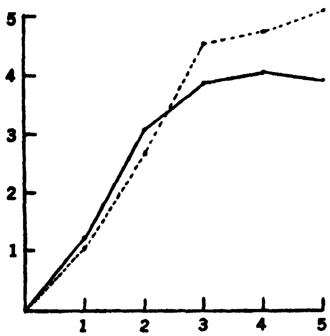


FIG. 1

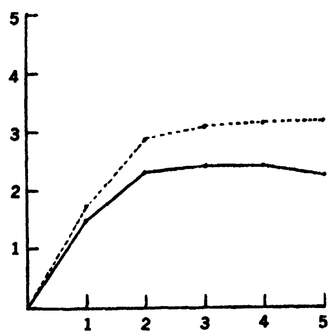


FIG. 2

FIGS. 1 AND 2.—Figure 1 is a graph of the data given in Table IV for the $5x$ concentrations. Figure 2 is a graph of the data in the same table for the $x$ concentrations. In each case the solid line represents the groups and the broken line the singles. Numbers on the ordinates denote the number of fissions, while those on the abscissas denote number of days.

Table V gives the results of a similar group of experiments carried for 7 days. In this case the average numbers of animals produced

TABLE V

7-DAY CULTURES IN $5x$ AND $x$ CONCENTRATIONS

| Days | No. of Animals in $5x$ Concentration | | | | | | No. of Animals in $x$ Concentration | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5 | 1 | 1 | 1 | 1 | 1 | 5 | 1 | 1 | 1 | 1 | 1 |
| 1........ | 17.0 | 4.3 | 3.5 | 3.3 | 3.0 | 2.5 | 21.3 | 6.8 | 5.3 | 5.0 | 4.8 | 3.5 |
| 2........ | 78.8 | 13.0 | 11.0 | 10.0 | 11.5 | 7.3 | 32.0 | 10.8 | 8.5 | 8.5 | 8.0 | 7.0 |
| 3........ | 106.0 | 21.5 | 18.3 | 13.8* | 15.3† | 16.3 | 32.3 | 13.5 | 10.3 | 10.0 | 9.5 | 9.0 |
| 4........ | 120.0 | 31.0 | 26.3 | 15.0 | 20.5 | 29.9 | 30.8 | 11.0 | 12.0 | 10.3 | 9.5 | 8.3 |
| 5........ | 162.5 | 53.0 | 41.3 | 19.0 | 33.5 | 45.0 | 27.0 | 11.0 | 12.0 | 12.3 | 9.5 | 8.3 |
| 6........ | 147.0 | 62.0 | 65.0 | 31.0 | 59.3 | 75.3 | 25.8 | 11.0 | 11.8 | 9.3 | 9.0 | 8.3 |
| 7........ | 135.2 | 69.0 | 61.0 | 35.0 | 70.8 | 86.3 | 22.5 | 11.0 | 11.8 | 8.5 | 8.5 | 8.3 |

* One culture died. † Two cultures died.

are given instead of the average fission rates. This table represents the average of four sets of five animals grouped and five animals isolated singly into 5 drops of both concentrations of bacteria. The whole series occupied 28 days. These data plotted in Figures 3 and 4 in terms of average fission rates will, perhaps, more clearly indicate the trends of these populations.

A comparison of the Figures 1 and 3 and 2 and 4 indicates that the same general relationships hold throughout, except that in the $5x$ concentrations shown in Figure 3 the singles did not surpass the groups during the third day as they had in the previous experiments. They did surpass the groups during the fourth day. The fact that

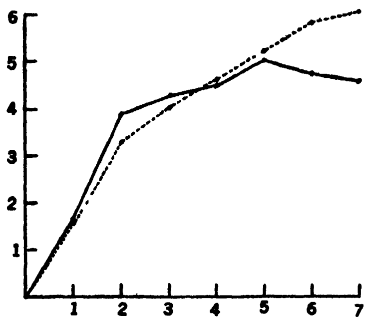


FIG 3

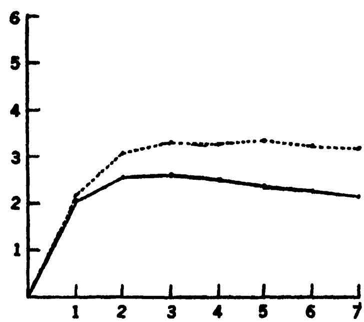


FIG 4

FIGS. 3 AND 4.—Figure 3 is a graph of the data given in Table V for the $5x$ concentrations, and Figure 4 is one of the data for the $x$ concentrations. The lines and numbers are used in the same way that they were used in Figures 1 and 2

three of the twenty single cultures died out during the third day in this series will probably account for this difference.

These studies on population trends in cultures of *Paramecium* show that in the $x$ concentrations the singles have a higher average rate of reproduction for the periods studied, although the difference during the first day is not significant. The highest rate of division for any one day in this density occurs during the first day in both the singles and the groups. The groups reach a maximum during the third day and then begin to decline in fission rate. The singles reach a maximum during the fifth day and then begin to decline.

There is little difference in the rate of reproduction of the groups and singles in $5x$ concentration for the first day. The highest rate of reproduction for any one day in the $5x$ concentrations occurs dur-

ing the second day in both the groups and the singles. The groups increase significantly faster than the singles during the second day in this density. During the third day the singles go ahead of the groups and remain so during the periods studied. (The exception to this, shown in Figure 3, seems to be due to the death of three of the cultures.)

Both singles and groups in the $x$ concentrations reproduce faster than those in the $5x$ concentrations during the first day. After the first day the groups in the $5x$ concentration have a much higher rate than those in the $x$ concentrations. After the second day the singles in the $5x$ concentrations have a higher fission rate than those in the less dense cultures.

The data given in Figures 3 and 4 show that all of the populations had reached a maximum and started to decline during the 7-day period except the singles in $5x$ concentrations. Two other experiments carried 8 and 9 days showed that such cultures would reach a maximum population by that time. In no case, however, did any culture starting with a single *Paramecium* reach a maximum as high as those maxima reached by the cultures starting with five animals. An examination of Table V brings this point out clearly.

## DISCUSSION

The finding of Hargitt and Fray (1917) that *Paramecium* will grow on a pure culture of *Bacillus subtilis* has been verified in these experiments. Other workers in this field have found that *Paramecium* will live on other kinds of bacteria in pure culture. The literature on this matter seems somewhat conflicting. Hargitt and Fray (1917) were unable to raise *Paramecium* on pure cultures of *Pseudomonas fluorescens*, but Oehler (1920) reported good cultures of *Paramecium* raised on this kind of bacteria. Chejfec (1929) raised *Paramecium* on cultures of *Bacterium coli*, but Phillips (1922) was unable to. In this work *Paramecium* did not grow long on suspensions of *Escherichia coli* or *Pseudomonas fluorescens*. In this connection Hetherington (1934), working with *Colpidium colpoda*, suggests that only particular strains of a species of bacteria may be suitable for a given protozoan. He says, "Indeed, the particular strain of the species used appears to be quite as important as the

larger group. Thus the coli strain from Stanford (*Escherichia coli communae*) and *Aërobacter aërogenes* 5 gave trouble, while *Escherichia communior* 1 and *A. aërogenes* from Stanford were as satisfactory as any bacterium tried."

Hetherington found that *Colpidium colpoda* was "not very particular with regard to the species of bacterium upon which it will grow." Penn (1934) in a discussion of various media suitable for the growth of the Protozoa says, "The question of food of Protozoa in most cases is not so specific. Any nutritive material admissible to the feeding apparatus of the organism concerned is usually satisfactory. The most important factor is the medium." The medium is important. And it appears that some of the smaller ciliates are not very selective in their food requirements. However, from many of the works already mentioned and from these experiments on *Paramecium* it appears that this form is selective in its food and that not any nutritive material admissible to its feeding apparatus is satisfactory. Johnson (1933) found that *Oxytricha fallax* was rather selective in its food.

Hetherington (1934) states that he obtained better cultures of *Colpidium* in nutrient media than he did in a salt solution. Phelps (1934), in his work with *Paramecium*, concluded that some organic matter other than the bacteria was essential in the medium. He found that *Paramecium* grew well on a culture of *Erythrobacillus prodigiosus* in a dried-lettuce medium. But he found that *Paramecium* would not grow on a culture of the same bacteria in a medium made by ashing dried lettuce leaves and dissolving the residue in distilled water. It may be that some kinds of bacteria work better than others as food for particular ciliates in non-nutrient media. Also, Phelps might have obtained different results using some other salt solution. In these experiments *Paramecium* maintained a high fission rate for a 51-day period in suspensions of *Bacillus subtilis* in a non-nutritive medium. Raffel (1930) working with *Paramecium*, Barker and Taylor (1931) working with *Colpoda*, and Johnson (1933) working with *Oxytricha* obtained similar results using non-nutritive media.

The results given in Tables III, IV, and V showing the fission rate of the groups in the $5x$ concentrations higher than the singles

in the same density during the second day are similar to those reported by Johnson (1933) for *Oxytricha* raised on suspensions of *Pseudomonas fluorescens*. These results may be interpreted as due to bacterial crowding. The groups are able to reduce the density of bacteria to a more optimal density faster than the singles, and thus show a higher rate of reproduction.

When the population trends in the different densities over the 3-, 5-, and 7-day periods are compared with those reported by Myers (1927) using hay medium, several differences are noted. The experiments reported here show that, within the limits used, the greater the density of bacteria the higher the maximum population reached with the same initial seeding in the same volume of medium. Myers found that the one- and two-animal cultures in 0.2-cc. hay medium arrived at the same maximum population, while the four-animal cultures had a lower maximum. In this work the one-animal cultures in 0.25 cc. of medium in both densities of bacteria never reached as high maximum populations as the five-animal cultures reached.

The highest rate of reproduction in these experiments occurred during the first and second days. There appeared to be plenty of bacteria in all of the cultures at the end of 7 days, even in the $x$ concentrations. That the decline in the fission rates and the failure of the singles to reach the maxima reached by the groups was not due to inadequate amounts of bacteria seemed certain. It appears that the presence of excretory substances will not explain all of the differences between the groups and the singles. Hetherington (1934) has suggested a possible reason for this difference. He says, "The bacteria removed from an agar plate by means of a platinum needle undoubtedly retain not only small amounts of metabolites but also some of the nutritive substances. This should provide for the continuance of more or less metabolism in Peter's medium." He found that washed *Bacillus niger* supported only a few divisions of *Colpidium;* that, unwashed, this bacterium, although giving no visible signs of metabolism, supported growth of *Colpidium* with a division rate of 2.70; and that the only bacterium clearly showing metabolism in the salt solution gave the highest division rate of any tried. He continues, "These results suggest that the consequences of the

metabolism of bacteria are crucial for the growth of *Colpidium colpoda*."

In view of the facts that the highest fission rates occurred during the first 2 days in these experiments, that there were still plenty of bacteria in the cultures at the end of 7 days, and that the rate of reproduction of the paramecia, even in the single-animal culture, did not increase proportionately after the second day, suggesting some change in the bacteria, it may be that Hetherington's suggestion is the explanation of these differences. Additional experiments must be carried out, using known bacteria in a nutritive medium, before further comparisons can be made between the type of population trends reported by Myers (1927) and those reported here.

## SUMMARY

1. Attempts were made to grow *Paramecium caudatum* in suspensions of *Escherichia coli*, *Aërobacter aërogenes*, *Pseudomonas fluorescens*, *Staphylococcus aureus*, *Serratia marcescens*, *Bacillus subtilis*, and an unknown yeast, and mixtures of all of these, in a non-nutritive salt solution.

2. Suspensions of *Bacillus subtilis* alone supported good growth of *Paramecium*. Over a 51-day period the average daily fission rate was 1.8.

3. Using five-animal and one-animal cultures in both $5x$ and $x$ concentrations of bacteria, both the groups and singles in the $x$ concentration had higher fission rates during the first day.

4. After the first day the groups in the $5x$ concentrations had higher reproductive rates than the groups in the $x$ concentrations.

5. After the second day the singles in the $5x$ concentration had a higher rate of division than those in the $x$ concentrations.

6. The singles in the $x$ concentrations reproduced significantly faster than the groups in the same concentration after the first day for the 7-day period.

7. The groups in the $5x$ concentrations reproduced significantly faster than the singles during the second day. After the third day the singles had a higher fission rate. The difference during the second day is interpreted as being due to bacterial crowding.

8. All of the cultures reached a maximum population during the

7-day period and started to decline, except the singles in the $5x$ concentrations.

9. The highest rates of fission in all of the cultures occurred during the first and second days.

10. In no case did any of the single-animal cultures reach as high a maximum population as did the group cultures. It is suggested that changes in the bacteria may be responsible for this.

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# EFFECTS OF HIGH VACUA AND EXTREME TEMPERATURES ON CYSTS OF COLPODA CUCULLUS[1]

(Five figures)

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DURING the spring and summer of 1931, investigations were undertaken on the effects of extreme temperatures on highly desiccated cysts.[2] Filter paper on which *Colpoda cucullus* had been induced to encyst was exposed to room humidity and temperature for several weeks and then cut into small rectangles. These rectangular pieces were carefully rolled, and to each piece was clamped a U-shaped segment of soft iron wire. Thereupon they were subjected for several days to high vacuum (about $10^{-5}$ mm. Hg) by means of a mercury pump which was provided with a liquid-air trap and an ionization gauge. Small pyrex tubes were sealed alternately to either side of a large extension tube on the high vacuum side of the mercury pump and kept at high temperature (450° C.) during the 2 or 3 days of evacuation. Into these small tubes, after cooling, the rolled pieces of filter paper were introduced with the aid of a magnet. The tubes were then sealed off.

Fifty-three such tubes which had been tested for high vacuum by means of an induction coil were placed from time to time into liquid air for periods from 1 hour to $12\frac{1}{2}$ days. After exposure, the tubes were broken and the small clamps were removed from the filter paper. These pieces of filter paper were thereupon immersed in balanced medium; and, with no exceptions, free-swimming *Colpoda* were observed in the medium in the course of several hours. Controls, of dishes and medium, were negative.

[1] The major portion of these experimental studies was supported through grants from the National Research Council and the Rockefeller Foundation, to whom we are duly grateful.

[2] Acknowledgment is made to Dr. W. C. Allee, of the Whitman Laboratory, and to Dr. H. B. Lemon, Department of Physics, University of Chicago, for generously providing facilities for the conduct of these preliminary experiments.

Similarly, cysts on filter paper which were air-dried for several weeks, but without evacuation, withstood direct exposure to liquid air. In some instances the cysts on filter paper were introduced directly into the liquid air; in other cases they were subjected by steps to the temperature of melting ice and of $CO_2$ ice and finally to that of liquid air. Conversely, the temperature for these cysts was raised by the same steps from liquid air to room temperature before inducing excystment. In all of these cases, also, free-swimming *Colpoda* were, in due course, found in the excystment medium.

Tubes containing the cysts on filter paper and sealed off under high vacuum were exposed for 11½ minutes to a temperature of 150° C. From these pieces of filter paper, free-swimming *Colpoda* were likewise afterward recovered, although, owing to the high vacuum in the tubes, it is not certain that the enclosed cysts themselves actually attained that extreme temperature.

These preliminary studies suggested the need of further work with improved methods, as applied especially to the thermal resistivity of cysts of *Colpoda* depending upon: (1) the rate of evacuation, (2) the degree of desiccation, (3) the rate of temperature change, and (4) the period of thermal exposure. The results of these later studies will now be presented.

## MATERIALS AND METHODS

When experimentally induced cysts of *Colpoda cucullus* are passing from the wet to the dried state (Taylor and Strickland), the most conspicuous feature is their great shrinkage in volume. Presumably, this is due to a rapid loss of water from the protoplasm and the cyst membranes. Similarly, von Brand (1924) observed that *Vorticella microstoma* loses about 50 per cent of its volume between the spherical maximally contracted free-swimming form and the cyst. The cysts of *Colpoda* which were in contact in the wet state are, in the air-dry state, separated by a space frequently greater than the radius of the dried cysts. The latter usually remain connected, however, by a bridge of the dried glutinous ectocyst.[3]

[3] It may be inferred that this glutinous membrane is comparable with that described and illustrated by Goodey (1913) as the "ectocyst." Weyer (1930) calls a similar structure in encysted *Gastrostyla steinii* the "Gallertartige Hülle" and applies the names "ectocyst" and "entocyst" to the intermediate and innermost membranes, such as may also be found in the cysts of *Colpoda cucullus*. Apparently an unfortunate confusion exists, therefore, in the usage of these terms.

Upon the addition of excystment medium it is but a matter of seconds for the dried cysts to swell to their former size. This rate of swelling is apparently independent of the age of the cysts. The time required for the completion of excystment, however, is increased about tenfold in the first six months, although the viability of the cysts (on Cellophane[4]) is decreased only about 15 per cent during that time (Taylor and Strickland).

The cysts of *Colpoda* may be kept for a very long (as yet undetermined) time in either the wet or dried state. Excystment was consistently induced for these experiments by washing and crowding the *Colpoda* (Barker and Taylor, 1931) by means of the centrifuge. A small drop of the concentrated culture was put into a sterile watch glass, and encystment followed in a few hours. Twenty-four hours later, 1 cc. of sterile balanced medium was added to each dish. If no organic solution is present, the cysts will remain quiescent indefinitely. With proper precautions, more balanced medium can be added as required. It should be noted, however, that an exceedingly small amount of fresh organic solute in such a small quantity of medium may effect partial or even complete excystment. In this paper we refer to cysts kept in this manner as "wet cysts." "Air-dry cysts," on the other hand, may be readily prepared on a Cellophane substrate according to the method described by Taylor and Strickland. By this convenient method, pieces of the Cellophane with a suitable number of dried cysts may be put into watch glasses or into glass tubes and thus subjected to experimental treatment as desired. The inertness and transparency of Cellophane afford an ideal substrate for quantitative, as well as qualitative, studies on the dried cysts of this invaluable ciliate.

Excystment was induced, throughout the following experiments, by a sterile solution of 1 gram of yeast extract paste in 1 liter of balanced salt medium.

For the production of high vacua a mercury diffusion pump and a Wegner-Deka-Micro fore pump[5] were used, finally assisted by a tube

[4] We gratefully acknowledge the generous supply of Cellophane and samples of material supplied by the Du Pont Cellophane Co., Inc., of New York.

[5] This high-vacuum equipment was provided us with the aid of funds contributed by the American Medical Association, for which acknowledgment is hereby gratefully made.

of activated charcoal immersed in liquid air. For convenience in handling in the treatments with high vacua and liquid air, the dry cysts on Cellophane were put into glass tubes of about 2 mm. internal diameter and 15 mm. in length. The one end of each tube was somewhat constricted; the other, lightly plugged with a few threads of cotton.

To determine the effect of heat on dry cysts, a thermostat-controlled electric oven was used. The bulb of the thermometer was in contact with the center of one of the watch glasses. In the case of wet cysts, the watch glass with the bulb of the thermometer in contact with it was completely immersed in water. In all cases the rate of rise in temperature was maintained as nearly as possible at 10° C. every 6 minutes.

## EXPERIMENTAL

### A. THE EFFECT OF RAPID EVACUATION OF DRIED CYSTS

Pieces from cyst-Cellophane preparations Nos. 371, 372, and 373 were used. The pressure was brought down very rapidly and was held at about $1\times10^{-5}$ atmospheres for 24 hours. Raising the pressure took about 5 minutes. The results are shown in Table I. The

TABLE I

MORTALITY DUE TO RAPID EVACUATION

| Preparation No. | Excystment | | Mortality (per Cent) |
|---|---|---|---|
| | $E_1$ Untreated (per Cent) | $E_2$ Rapid Evacuation (per Cent) | |
| 371 | 73 | 39 | 46.6 |
| 372 | 64 | 37 | 42.2 |
| 373 | 68 | 36 | 47.1 |

mortality is expressed $(E_1-E_2)/E_1\times100$. Many shattered cysts, due to the rapid fall of pressure, were visible under the microscope.

### B. THE EFFECT OF GRADUAL EVACUATION OF DRIED CYSTS

The pressure was decreased by steps for 4 days until approximately $1\times10^{-5}$ atmospheres were reached, and maintained around that point for 3 days more. The pressure was then slowly raised, by va-

rious devices in the course of a further 3 days, to atmospheric pressure. Table II and Figure 1 show the effect produced on the percentage and rate of excystment. These results show that viability is not affected by gradual evacuation. It is only a coincidence that

TABLE II

THE EFFECT OF GRADUAL EVACUATION OF DRIED CYSTS

| Air-dry Untreated | | Air-dry Evacuated | |
|---|---|---|---|
| Time (Hours) | Excystment (per Cent) | Time (Hours) | Excystment (per Cent) |
| 0 | 0 | 0 | 0 |
| 2¼ | 8.6 | 2 | 2.6 |
| 2¾ | 38.7 | 2½ | 15 |
| 3¼ | 49 | 3 | 32 |
| 3¾ | 55 | 3½ | 41 |
| 4¼ | 60 | 4 | 48 |
| 4¾ | 62 | 4½ | 53 |
| 5¼ | 64 | 4¾ | 56 |
| 5¾ | 66 | 5¼ | 60 |
| 20 | 71 | 20 | 71 |

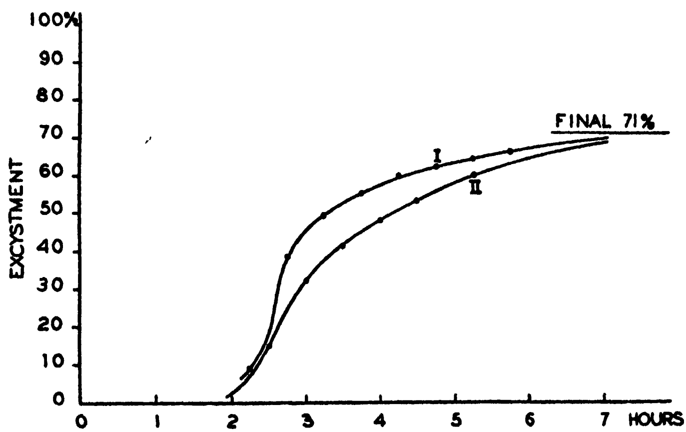


FIG. 1.—The effect of gradual evacuation of dried cysts. *I*, untreated; *II*, evacuated

the untreated and the evacuated cysts gave in this instance the same total excystment (71 per cent). The final count at 20 hours was made many hours after excystment was actually completed. The average final excystment in all (sixteen) tests with the untreated material

was 76.4 per cent; in all (ten) tests with the evacuated material, it was 76.7 per cent.

### C. A COMPARATIVE STUDY OF THE EFFECT OF EXPOSURE TO HIGH TEMPERATURE ON WET AND DRY CYSTS

*a) Momentary exposure.*—At certain temperature intervals a watch glass was removed from the oven. Table III and Figures 2 and 3 show the results obtained. From Figures 2 and 3 it appears that the lethal temperature for wet cysts is 51° C. and for air-dry cysts 121°. It should be noted, however, that these "air-dry" cysts are

TABLE III

EFFECT OF MOMENTARY EXPOSURE

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Wet cysts:* | | | | | | | | | | | | |
| Temperature (°C.).. | 34 | 44 | 45 | 49 | 50.5 | 51 | 52 | | | | | |
| Excystment (per cent)........... | 100 | 100 | 98 | 94.7 | 88 | 78 | 0 | | | | | |
| *Dry cysts:* | | | | | | | | | | | | |
| Temperature (°C.).. | 35 | 50 | 65 | 75 | 80 | 85 | 90 | 97 | 105 | 110 | 118 | 125 |
| Excystment (per cent)........... | 98 | 97 | 98 | 91 | 94 | 93 | 92 | 97 | 93 | 81 | 66 | 0 |

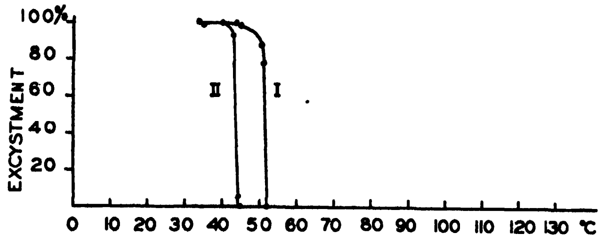


FIG. 2.—Effect of high temperatures on wet cysts. *I*, momentary exposure; *II*, exposure for 1 hour.

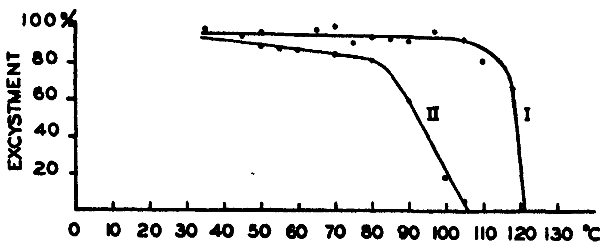


FIG. 3.—Effect of high temperatures on dry cysts. *I*, momentary exposure; *II*, exposure for 1 hour.

no longer in equilibrium with the water-vapor tension of the laboratory but have become progressively drier as the temperature increased.

*b*) *Exposure for 1 hour.*—In the case of each watch glass the temperature was raised at the usual rate from laboratory temperature to the desired level, and maintained at that point for 1 hour. Table IV and Figure 3 show the results obtained.

TABLE IV

EFFECT OF EXPOSURE FOR 1 HOUR

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Wet cysts*: | | | | | | | | |
| Temperature (°C.)... | 35 | 40 | 43 | 44 | 45 | 50 | 60 | |
| Excystment (per cent) | 98.6 | 100 | 93 | 5.8 | 0 | 0 | 0 | |
| *Dry cysts*: | | | | | | | | |
| Temperature (°C.)... | 50 | 60 | 70 | 80 | 90 | 100 | 105 | 110 |
| Excystment (per cent) | 89 | 86.4 | 85.6 | 82 | 59 | 18 | 5.5 | 0 |

As indicated by Figures 2 and 3 it seems that the lethal temperatures for exposure for 1 hour are for wet cysts 44°; for dry cysts, 106° C. In this case also, the "air-dry" cysts became drier, owing to the rising temperature and the duration of exposure.

### D. THE EFFECT OF PROLONGED EXPOSURE TO 70° C.

Some pieces of cyst-Cellophane preparation No. 607 were put into a sterile Petri dish. Similar pieces, each with about five hundred cysts, were put into watch glasses. The temperature of the oven was raised at the usual rate to 70° C. and maintained continuously at that point for 26 hours. At intervals a watch glass was removed. After 8 hours the Petri dish was removed. Table V and Figure 4

TABLE V

EFFECT OF PROLONGED EXPOSURE TO 70° C.

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Time (in hours)........... | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 10½ |
| Excystment (per cent)..... | 85.4 | 80.6 | 62.2 | 67.4 | 66.7 | 69.7 | 60.0 | 80 | 72 |
| Time (in hours)........... | 12 | 13½ | 15 | 16½ | 18 | 20 | 22 | 24 | 26 |
| Excystment (per cent)..... | 72.5 | 90 | 72.5 | 67 | 74.5 | 98.5 | 90.4 | 80 | 73 |

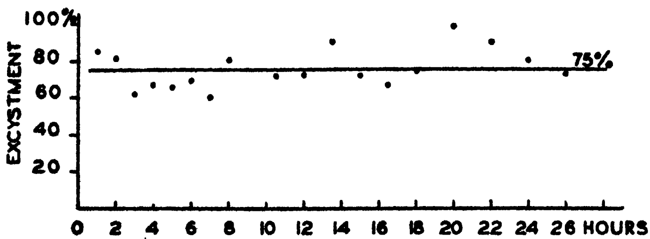


FIG. 4.—Exposure to 70° C. for long period

show the excystment obtained from the cysts in each watch glass.

In order to find out whether absolutely sterile *Colpoda* in large numbers could be obtained in this way, the pieces in the Petri dish were tested for aërobic and anaërobic bacteria and were found to be sterile.

### E. THE EFFECT ON DRY CYSTS OF PROLONGED IMMERSION IN LIQUID AIR

Preparation No. 607 was used. The liquid air evaporated continuously during the experiment, so that the temperature was pre-

TABLE VI

EFFECT OF PROLONGED IMMERSION IN LIQUID AIR

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Time (in hours)............ | 1 | 2 | 4 | 6 | 8 | 11½ | 13½ |
| Excystment (per cent)...... | 88 | 79.3 | 61.3 | 69 | 77.8 | 84.5 | 81.2 |

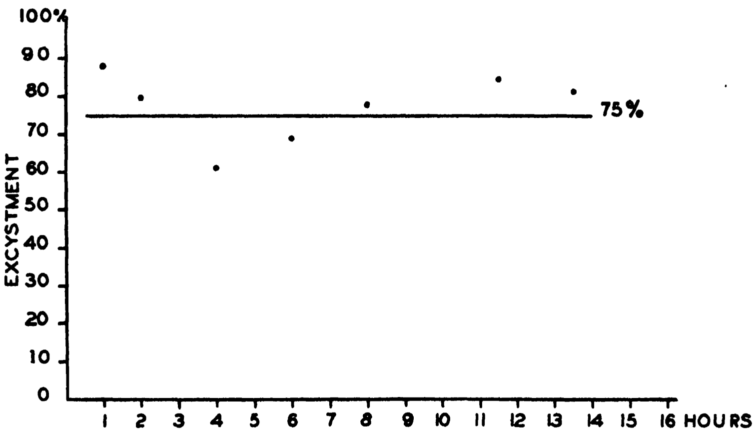


FIG. 5.—Effect of prolonged immersion in liquid air

sumably around −180° C. The capsules were dropped into the liquid air. At intervals a capsule was removed; in about 2 minutes it and the cysts in it had reached the temperature of the laboratory. Table VI and Figure 5 show the excystment obtained. The average excystment was approximately normal for that preparation at the date of these experiments. From the results noted in Table VI, it is evident that these exposures to the temperature of liquid air had no significant effect upon the percentage and rate of subsequently induced excystment.

## DISCUSSION

For certain experimental work it would be most valuable to have protoplasm without free (i.e., unbound) water. It seems probable that a high vacuum would be a convenient and effective means toward this end, even though the actual degree of desiccation (water-vapor tension of the protoplasm) cannot be accurately determined.

It is evident from the results obtained by rapid evacuation that protoplasm in equilibrium with the humidity of the laboratory has still much free moisture in it. Later experiments have shown that the rapid rise to atmospheric pressure does not have any injurious effect.

Gradual evacuation does not affect the viability of the protoplasm; but, presumably correlated with increased desiccation, the rate of excystment is at first less for the evacuated than for the untreated cysts. For the example shown in Figure 1, at time 2 hours and 30 minutes the ratio is 1:1.87. Goodey (1913) found that, if cysts of *Colpoda* are kept dried on filter paper for some weeks, their power of excysting rapidly is considerably diminished, though he has no quantitative data. He says, however, that in his tests on the effect of temperature (20°–40° C.) on excystment, at 20° C. a few were active after 2 hours, 12 minutes. This agrees with the curve in Figure 1 for which the temperature is 20°–22° C. and the age of the cyst preparation 36 days.

When the cysts of *Colpoda cucullus* are passing from the wet to the air-dry state, the loss of water is considerable and very rapid. If it is assumed that the temperature of coagulation of a protein in colloidal solution varies with the amount of free water, a comparison of these critical temperatures for wet and air-dry cysts should give some idea of the relative desiccation of the protoplasm. Barker (1933) determined the temperature of denaturation of crystallizable egg albumin at various relative humidities in equilibrium with various water-vapor pressures. Our dry cysts, at the start of the experiment in equilibrium with the humidity of the laboratory, gave 50 per cent excystment after 1 hour at 92° C. Assuming that Barker's results may be reasonably compared with ours, and that an excystment of 50 per cent of the normally viable cysts corresponds

approximately to the mid-point of the denaturation temperature range of albumin, we find from Barker's Table II that the relative humidity should be 58 per cent. The humidity of the laboratory was not measured at the time of our experiments, but this value (58 per cent) for the laboratory seems reasonable enough.

Experiments to determine the effect of high temperatures on the dried cysts in equilibrium with constant and definite water-vapor pressures are now in progress.

For wet cysts the lethal temperature, corresponding possibly to the coagulation temperature of the whole or of some important constituent of the protoplasm at that degree of hydration, is 51.1° C. Goodey states that cystase, the enzyme which he says dissolves the cyst membrane to release the protozoon, is inactivated at 40° C.; but we find that at this temperature, even after 1 hour, excystment seems to be 100 per cent. It may be that, if an enzyme is required to assist the violent exertions of the protozoon to rupture the endocyst, the enzyme is produced only in its heat-sensitive form at the time it is required. He finds that there is no excystment at 40° C. but does not say how long the cysts were subjected to this temperature. It seems probable that it was for several hours. For a period of 1 hour the lethal temperature seems to be about 44° C.

Air-dried cysts on a Cellophane substrate have evidently lost a large part of their free water, as the protoplasm is almost unaffected at 110° C. and may even survive progressive heating to and momentary exposure to 120°. About 20 per cent of the dry cysts can stand the boiling-point of water for an hour. The slope of the curve for 1 hour's exposure (Fig. 3) is an indication of the progressive desiccation of the dry cysts with time and rising temperature.

In this connection it is interesting to note that in 1865 Meunier, "to throw new light on certain cases of spontaneous generation claimed to have occurred in boiled infusions," boiled some hay dust containing cysts of *Colpoda* and found that they did not survive the experience. Dawson and Hewitt (1931) induced excystment of *Colpoda* from dry hay dust after 5 years and 4 months. The time required for excystment was 18–24 hours.

From Figure 4 it is evident that exposure to a temperature of 70° C. for 26 hours does not injure the cysts. After 8 hours' heating,

the cysts in the Petri dish were sterile. No precautions had been taken to protect preparation No. 607 from contamination. It had been handled, cut with unsterilized instruments, and frequently exposed to the air. For some experimental purposes it is important to have absolutely sterile protozoa. It is claimed not that a cyst preparation can be sterilized in this manner under all circumstances but that this preparation, from a culture fed with a non-spore-forming bacterium, was completely sterile after heating as described.

Luck and Sheets (1931) made extensive efforts to sterilize cysts by chemical reagents but without success. Oehler (1924) also tried chemical means of sterilization without success. He was able to obtain sterile *Colpoda* from cysts, dried presumably in the detritus of the medium, which were kept at 37° C. for 6 weeks. He was also able to sterilize cysts, which he had raised in a pure culture of *Saccharomyces exiguus*, by heating them for 24 hours at 64° C.

From Section E it appears that prolonged exposure to intense cold has no injurious effect. The average excystment after 13½ hours in liquid air is 78 per cent, which is only about 4 per cent less than the expected average excystment of the untreated preparation at that date and is within the range of variations expected. Excystment was complete after 8 hours, which is little, if any, more than is required for the untreated cysts (65 days dry). On immersion the cysts must have been frozen instantly. Nor does it seem that a rise in temperature of about 200° in a few minutes has any injurious effect. It is known that sudden freezing with intense cold causes the formation of smaller crystals, consequently with less tendency to disrupt tissues on freezing or thawing.

It would appear that the protoplasm adjusts its water content, more or less rapidly according to circumstances, to the humidity of the environment; and that the effect of such external conditions as high vacua and extreme temperatures on the viability of the protoplasm may be correlated with the degree of its desiccation.

## SUMMARY

Experiments on the viability of wet and dried cysts of *Colpoda cucullus* under various conditions are described:

1. The mortality due to very rapid evacuation under the conditions of the experiment was found to be about 46 per cent.

2. The effect of gradual evacuation on viability was found to be nil; there was a noticeable decrease in the rate of excystment.

3. The effect of high temperatures on wet and dry cysts is shown, and the lethal temperatures determined for momentary exposure and exposure for 1 hour.

4. The viability of the cysts is not impaired by exposure to 70° C. for 26 hours, and the cyst-cellophane preparation under test was completely sterile after 8 hours.

5. Intense cold for long periods, such as immersion in liquid air for 13½ hours, does not affect the viability of the cysts; some excystment (percentage unknown) was obtained from cysts exposed to liquid-air temperature for 12½ days.

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# THE EFFECT OF DESICCATION ON SURVIVAL AND METAMORPHOSIS OF THE JAPANESE BEETLE (POPILLIA JAPONICA NEWMAN)

(One figure)

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INSECTS manifest considerable variation in their ability to survive desiccation. Breitenbecher (1918) reported that the June beetle, *Lachnosterna*, could not survive a loss by desiccation of 15 per cent of its original weight; whereas, the beetle *Cotalpa lanigera* could survive a loss of 25 per cent, and the potato beetle, *Leptinotarsa decemlineata*, more than 50 per cent.

Insects which live in a comparatively dry medium and feed on dry food seem to possess the capacity to conserve body water. In many of these insects, desiccation may result in a loss of considerable body weight before any change in water content is noted. Thus, Berger (1907) showed that the larvae of *Tenebrio molitor* could be kept in an absolutely dry medium and that, in spite of a great loss of water, the relative fluid content of the body remained approximately constant. Similar results were obtained by Hall (1922) and by Buxton (1930) with *Tenebrio* larvae. The former found that these larvae could be desiccated to 52.6 per cent of their weight without losing vitality; and the latter, that they maintained their original ratio of water to dry matter during a month of starvation at humidities ranging from 0 to 60 per cent. This ability of *Tenebrio* larvae to maintain a constant water content during periods of desiccation is probably due to the utilization of metabolic water, as has been shown by Mellanby (1932*a*). Speicher (1931) placed the second- and third-instar larvae of the Mediterranean flour moth, *Ephestia kuehniella*, in jars containing anhydrous $CaCl_2$ and fed them meal previously dried at 103° C. Despite the dry atmosphere, the larvae and pupae maintained a constant percentage of free water in their bodies as long as they survived. Buxton (1932) observed that the bug *Rhodnius prolixus* lost dry weight and water in the same proportion when kept in dry air even when a weight loss of 50 per cent had resulted.

On the other hand, the larva of the Japanese beetle normally lives in moist soil, where it usually is not subjected to the drying effects of the atmosphere. Therefore a mechanism for the conservation of water is probably not necessary, and this larva might be expected to react to desiccation in a manner different from that of insects living in dry air. For this reason it was thought desirable to determine the rate and manner of desiccation, and the vital limits of desiccation, of the various stages in the life-cycle of this beetle.

Ludwig (1931) found that during the normal metamorphosis of the Japanese beetle from larva to adult there is a loss of weight amounting to one-half of the maximum larval weight, and that during the emergence of the adult the loss of weight amounts to one-third of the pupal weight. The water content also was found to decrease from 78.0 per cent in the larva to 66.6 per cent in the adult. Hence, this weight loss is due largely to a loss of water. Since dehydration normally occurs during the transformation from larva to adult, the question arises as to whether it is merely incidental or is actually essential to the metamorphic changes. Experiments were therefore performed to determine the effects of partial desiccation on the loss of weight and water occurring during metamorphosis.

## MATERIAL AND METHODS

The Japanese beetles used in these experiments were obtained from two sources. (1) The third-instar larvae were collected in the field during the winter and spring months and were secured from Dr. Henry Fox, of the Japanese Beetle Laboratory, Moorestown, New Jersey. Prepupae and pupae were obtained from these larvae. (2) The first- and second-instar larvae developed from eggs collected in the laboratory. Each individual was kept in a 1-ounce metal salve box containing moist plant mold. The humidity of the plant mold was kept near the point of saturation by the addition of tap water. Each larva was also given several grains of wheat to serve as additional food. Throughout the feeding period, an abundance of food was always available, except during the period of desiccation, when the larvae were starved.

Desiccation experiments were performed on the first-, second-, and third-instar larvae, early prepupae, late prepupae, and early pupae.

The early prepupa differs from the third-instar larva only in the creamy-white color of its body, due to the elimination of fecal material. In order to obtain comparable results, desiccation experiments were always begun on the first day the individual was definitely recognized as being a prepupa. The late prepupa is distinguished by the degeneration of the legs and mouth parts and the shrinking of the body within the larval skin. Desiccation experiments on the pupae were always started the first or second day after pupation.

In all cases the beetles were kept at room temperature (18°–25° C.) until used in the desiccation experiments. Two groups of experiments were performed. In the first group, a relatively slow desiccation was obtained by placing each beetle in a small, unstoppered vial kept in an incubator at approximately 25° C. First-, second-, and third-instar larvae, early prepupae, late prepupae, and early pupae were desiccated in this manner. During the course of preliminary experiments, it was found that larvae lost weight much more rapidly when placed in metal salve boxes than when placed in glass vials. Consequently, a second group of experiments was devised in which a more rapid desiccation of third-instar larvae was obtained by placing each individual in an uncovered, 1-ounce metal salve box in an incubator at 25° C. The relative humidity of the incubator usually ranged from 30 to 35 per cent. Weight readings were made daily; and when desiccation to the desired degree was obtained, the beetles were returned to the salve boxes containing moist plant mold and kept at 25° C. for further observations. Fecal material was always weighed along with the larva. Daily observations were made on all desiccated beetles, and weight readings were made on the larvae at least twice a week. An individual was considered to have recovered when it had regained its original weight or when it had transformed normally to the succeeding stage. Following desiccation, weight readings were made on each individual when it had reached the following stages: late prepupa; early pupa; and adult, within 24 hours of emergence. All weighings were made on a chainomatic balance sensitive to 0.1 mg.

Readings on the water content of 35 normal and 33 partially desiccated third-instar larvae were made to determine whether all of the loss of weight was due to the evaporation of water. Since this proved

to be the case, determinations of water content of normal individuals in the early prepupal, late prepupal, and early pupal stages were made for the purpose of calculating the water content of individuals when they had reached the maximum fatal limits of desiccation. Water content of adults, obtained from prepupae and pupae which had been previously desiccated, was determined for comparison with similar readings obtained on adults which had not been previously desiccated. To determine water content, each individual was first weighed, then dehydrated in an oven at approximately 60° C. until its weight remained constant, and finally weighed again. The difference between the two readings is interpreted as the weight of the water lost by evaporation.

## OBSERVATIONS

Within each stage of the life-cycle there is a tremendous variation in the rate at which desiccation occurs. For instance, first-instar larva No. 24 became desiccated to 51.6 per cent of its original weight in 23 hours, while first-instar larva No. 12 required 142 hours to become desiccated to 54.1 per cent of its original weight. These two larvae were dehydrated at the same time, so that conditions of temperature and humidity were identical. Similar variations were found in each stage of development. Figure 1 shows that, under approximately identical conditions, the various stages lose weight at different rates. The first instar, which is the smallest larval instar and with relatively the greatest amount of surface, loses weight most rapidly. The other stages follow in the order of development and metamorphosis, with the exception of the late prepupa, probably because a rapid loss of weight normally occurs in this stage. The graphs in this figure represent averages taken from records of 35 or more individuals of each stage. The average rate of desiccation gradually decreases as the water content of the beetles decreases. This is not very pronounced in the late prepupal stage, probably also because of the rapid loss of weight characteristic of this stage.

Tables I and II show the maximum fatal limits of desiccation, and the percentage of survivors above this limit, for each stage of development and metamorphosis. The larval stages are the most resistant; the first instar survives a desiccation to 50 per cent of its

original weight; the second instar, to 45 per cent; and the third instar, to 50 per cent. Not only is the second instar able to withstand a greater loss of weight, but the percentage of survivors at points above this fatal limit are greater than in the other larval stages. One

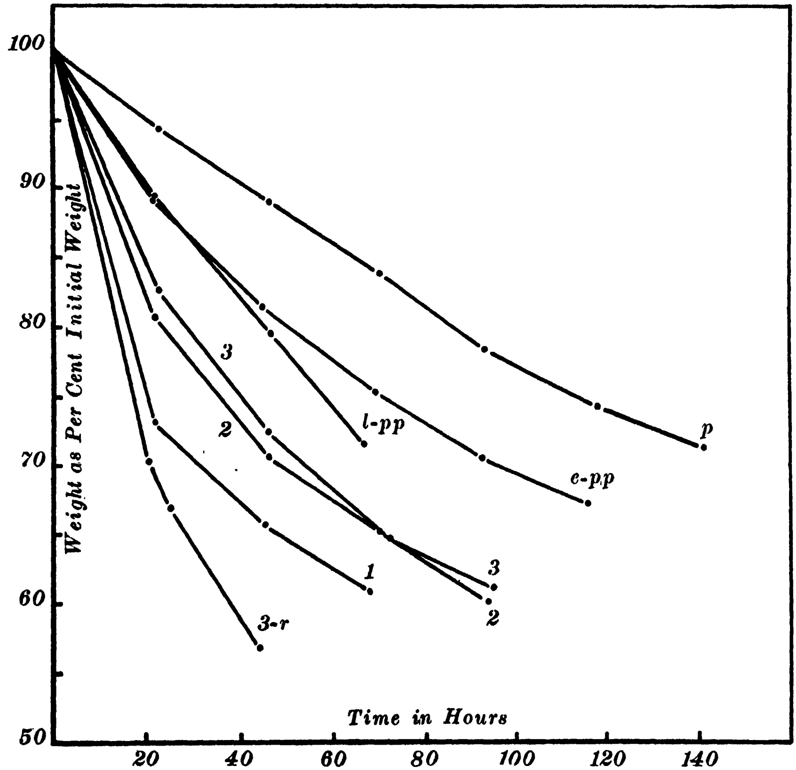


FIG. 1.—Rate of weight loss during desiccation of various stages: *1*, first-instar larva; *2*, second-instar larva; *3*, third-instar larva; *3-r*, third-instar larva, rapid desiccation; *e-pp*, early prepupa; *l-pp*, late prepupa; *p*, early pupa.

hundred per cent of the second-instar larvae survived a weight loss to 56 per cent of their original weight. In the first instar, 71.4 per cent survived the same amount of desiccation; and in the third instar, only 66.6 per cent survived.

Rapid desiccation is more harmful than slow desiccation. In the rapid desiccation of the third instar, the maximum fatal limit was

TABLE I

DESICCATION AND SURVIVAL OF THE LARVA OF THE JAPANESE BEETLE

| Extent of Desiccation (Weight, Percentage of Original Weight) | Number of Larvae | Percentage of Survivors |
|---|---|---|
| | FIRST-INSTAR LARVA DESICCATED | |
| Less than 50 | 6 | 0.0 |
| 50–52 | 15 | 20.0 |
| 52–54 | 21 | 38.0 |
| 54–56 | 18 | 44.4 |
| 56–58 | 21 | 71.4 |
| | SECOND-INSTAR LARVA DESICCATED | |
| 45–48 | 15 | 13.3 |
| 48–50 | 10 | 40.0 |
| 50–52 | 20 | 35.0 |
| 52–54 | 21 | 66.6 |
| 54–56 | 23 | 91.3 |
| 56–58 | 25 | 100.0 |
| | THIRD-INSTAR LARVA DESICCATED | |
| Less than 50 | 10 | 0.0 |
| 50–52 | 24 | 8.3 |
| 52–54 | 25 | 20.0 |
| 54–56 | 24 | 50.0 |
| 56–57 | 12 | 66.6 |
| 57–65 | 15 | 73.3 |
| 65–70 | 13 | 100.0 |
| | THIRD-INSTAR LARVA DESICCATED (RAPID DESICCATION) | |
| Less than 50 | 13 | 0.0 |
| 50–52 | 6 | 0.0 |
| 52–54 | 17 | 0.0 |
| 54–56 | 21 | 14.2 |
| 56–58 | 24 | 29.1 |
| 58–59 | 11 | 45.4 |

54 per cent of the original weight; and only 29.1 per cent survived a weight loss to 56 per cent of their original weight.

As the individual metamorphoses, it becomes less resistant to desiccation; the fatal limits are 56 per cent of original weight in the

TABLE II

DESICCATION AND SURVIVAL OF PREPUPAE AND PUPAE OF THE JAPANESE BEETLE

| Extent of Desiccation (Weight, Percentage of Original Weight) | Number of Beetles | Percentage To Form Pupae | Percentage To Form Normal Adults |
|---|---|---|---|
| | EARLY PREPUPA DESICCATED | | |
| 54–56 | 9 | 0.0 | 0.0 |
| 56–58 | 22 | 13.6 | 9.0 |
| 58–60 | 20 | 25.0 | 15.0 |
| 60–62 | 20 | 40.0 | 40.0 |
| 62–64 | 20 | 75.0 | 60.0 |
| 64–66 | 20 | 70.0 | 50.0 |
| | LATE PREPUPA DESICCATED | | |
| 64–67 | 6 | 0.0 | 0.0 |
| 66–69 | 6 | 16.6 | 0.0 |
| 69–71 | 8 | 25.0 | 0.0 |
| 71–73 | 8 | 62.5 | 25.0 |
| 73–75 | 8 | 87.5 | 50.0 |
| | EARLY PUPA DESICCATED | | |
| 64–67 | 21 | ..... | 0.0 |
| 67–69 | 20 | ..... | 0.0 |
| 69–71 | 20 | ..... | 5.0 |
| 71–73 | 20 | ..... | 15.0 |
| 73–75 | 20 | ..... | 20.0 |
| 75–77 | 20 | ..... | 25.0 |
| 77–79 | 20 | ..... | 70.0 |
| 79–81 | 20 | ..... | 60.0 |

early prepupal stage, 66 per cent in the late prepupal stage, and 69 per cent in the pupal stage. It is interesting to correlate the slower rate of drying of the pupal stage (Fig. 1) with its higher fatal limit of desiccation (Table II). It appears that, although the pupae are

not able to survive a loss of more than 31 per cent of their weight, they are still able to withstand exposure to drying environmental conditions as long as, or longer than, the other stages, because of the slow rate of water loss characteristic of this stage.

Approximately all of the weight loss in the Japanese beetle is due to the evaporation of water. The average water content of 35 third-instar larvae was found to be 81.0 per cent. Using this figure as normal, the amount of water in partially dehydrated larvae was calculated by assuming that all of the weight lost was due to the loss of water. Thirty-three such partially dehydrated larvae were then completely desiccated, and the observed water content compared with that calculated for the same individuals. The average deviation was 0.4 per cent. Hence, there is evidently no conservation of metabolic water in the Japanese beetle larva.

The water content of the various stages of metamorphosis varied as follows: third-instar larva, 81.0 per cent; early prepupa, 77.1 per cent; late prepupa, 75.9 per cent; and early pupa, 76.5 per cent. Using these figures, the water content was calculated for individuals of various stages when they had reached the maximum fatal limits of desiccation. At this point the third-instar larva contained an average of 62.0 per cent water; the early prepupa, 59.1 per cent; the late prepupa, 63.4 per cent; and the early pupa, 65.9 per cent. When degree of desiccation is measured by residual water content, there is less variation than when measured by total weight loss. When measured by weight, as percentage of original weight, the maximum fatal limits of desiccation varied from 45 per cent in the second instar larva to 69 per cent in the early pupa; whereas, when measured by water content, they varied from 59.1 per cent in the early prepupa to 65.9 per cent in the early pupa.

Table III gives a comparison of the weight loss and water loss occurring during normal metamorphosis and during metamorphosis when early prepupae and early pupae were partially desiccated. The figures in the first column are taken from Ludwig (1931). They show a weight loss of 23.6 mg. in male and 30.2 mg. in female beetles during the change from early prepupa to early pupa. During normal emergence of male and female beetles, there is also a weight loss of 66.5 mg. and 76.1 mg., respectively. When the early prepupae were

desiccated (second column of Table III), the average weight loss due to desiccation of the survivors was 81.7 mg. in male and 99.1 mg. in female beetles, while in the same group the weight loss during the

TABLE III

COMPARISON OF METAMORPHIC CHANGES IN DESICCATED AND NORMAL JAPANESE BEETLES

(Data for normal animals, duration of pupal stage excepted, are from Ludwig, 1931)

(Weight expressed in milligrams)

| | Normal (Ludwig, 1931) | Prepupae Desiccated | Pupae Desiccated | |
|---|---|---|---|---|
| | | | Normal Emergence | Abnormal Emergence |
| Number of beetles used: | | | | |
| Males | 29 | 22 | 25 | 56 |
| Females | 27 | 15 | 45 | 51 |
| Weight of early prepupae: | | | | |
| Males | 205.5±2.7 | 216.0±2.48 | ........... | ........... |
| Females | 253.7±3.4 | 272.7±3.71 | ........... | ........... |
| Weight of early pupae: | | | | |
| Males | 181.9±2.5 | 165.4±2.24 | 183.1±2.48 | 187.6±1.75 |
| Females | 223.5±3.9 | 209.0±3.51 | 209.8±2.84 | 225.4±2.25 |
| Weight loss due to desiccation of early prepupae: | | | | |
| Males | ........... | 81.7 | ........... | ........... |
| Females | ........... | 99.1 | ........... | ........... |
| Weight loss, early prepupa to early pupa: | | | | |
| Males | 23.6 | 50.6 | ........... | ........... |
| Females | 30.2 | 63.7 | ........... | ........... |
| Weight of adults: | | | | |
| Males | 114.3±1.9 | 107.0±1.72 | 111.3±2.05 | 131.3±1.77 |
| Females | 146.3±2.6 | 140.3±2.88 | 128.1±2.16 | 158.0±2.04 |
| Weight loss due to desiccation of early pupae: | | | | |
| Males | ........... | ........... | 37.0 | 54.2 |
| Females | ........... | ........... | 41.7 | 63.2 |
| Weight loss on emergence: | | | | |
| Males | 66.5 | 58.9 | 34.7 | 2.0 |
| Females | 76.1 | 70.1 | 39.5 | 4.1 |
| Percentage of water in adults | 66.6±0.62 | 66.9±0.44 | 68.0±0.29 | ........... |
| Duration of pupal stage (days) | 9.75±0.04 | 10.5±0.06 | 11.0±0.06 | 12.1±0.05 |

change from early prepupa to early pupa was only 50.6 mg. and 63.7 mg., respectively. Thus there appears to be a partial recovery of water, amounting to more than 30 mg., during this stage of metamorphosis. Normally, this stage is characterized by a slight loss of

water. In this series, 58.9 mg. and 70.1 mg. were lost during the emergence of male and female beetles, respectively, the adults possessing the normal water content of 66.9 per cent.

When early pupae were desiccated (third column of Table III), the emergence of normal adults was accompanied by a loss of only 34.7 mg. and 39.5 mg. in male and female beetles, respectively. In this series, 37.0 mg. and 41.7 mg. were lost by desiccation. Hence, the total weight loss from early pupa to adult was 71.7 mg. in the males and 81.2 mg. in the females. Practically no recovery of lost water occurred during the pupal stage. In this series, the water content of the adult beetles averaged 68.0 per cent. Therefore, regardless of whether the prepupae or pupae were desiccated, the normal water content of the adult was restored by a loss of less water at the time of emergence. When the larvae were desiccated, complete recovery of lost water was necessary before development could proceed to the next stage.

The fourth column of Table III gives the results obtained when pupae were desiccated too far to permit them to develop into normal adults. The pupae seemed to develop normally, the adults forming within the pupal skin. The color of the head, thorax, and legs changed to the dark green characteristic of normal pupae before emergence. However, the only change which occurred at the time of emergence was the movement of the legs which resulted in freeing them from the pupal skin. All other parts of the body remained within the pupal skin. A similar condition was described by Ludwig (1928) for pupae subjected to extremes of temperature. Table III shows that on the emergence of these abnormal adults practically no water was lost.

Another important effect of desiccation was to increase the duration of the pupal stage (transverse row at the bottom of Table III). At 25° C. the pupal stage in the normal individual requires $9.75 \pm 0.04$ days. When early prepupae were desiccated, it required $10.5 \pm 0.06$ days. In this series there had been a partial recovery before pupation. When early pupae were desiccated, the pupal stage required $11.0 \pm 0.06$ days in those cases where normal adults were produced and $12.1 \pm 0.05$ days when abnormal adults resulted. In the last group there had also been a much greater water loss.

The time required for recovery from desiccation in third-instar larvae was correlated with original weight, total loss of water, and rate of desiccation. These observations are summarized in Table IV. They indicate that the greater the degree of desiccation, the

TABLE IV

EFFECT OF VARIOUS FACTORS ON THE TIME FOR RECOVERY FROM DESICCATION OF THE THIRD-INSTAR LARVA

DEGREE OF DESICCATION

| Extent of Desiccation (Weight, Percentage of Original Weight) | Number of Larvae | Average Time for Recovery at 25° C. (Days) | Min. Time (Days) | Max. Time (Days) |
|---|---|---|---|---|
| 50–55 | 14 | 22.5 | 14 | 30 |
| 55–60 | 21 | 18.7 | 8 | 32 |
| 60–65 | 12 | 14.5 | 6 | 27 |
| 65–70 | 14 | 8.7 | 3 | 19 |

WEIGHT OF LARVAE

| Weight of Larvae (Mg.) | Number of Larvae | Average Time for Recovery at 25° C. (Days) | Min. Time (Days) | Max. Time (Days) |
|---|---|---|---|---|
| Less than 200 | 20 | 15.2 | 3 | 29 |
| More than 200 | 41 | 17.1 | 3 | 32 |

TIME OF DESICCATION

| Time for Desiccation (Hours) | Number of Larvae | Average Time for Recovery at 25° C (Days) | Min. Time (Days) | Max. Time (Days) |
|---|---|---|---|---|
| Less than 72 | 33 | 14.0 | 3 | 27 |
| 72–100 | 16 | 15.4 | 6 | 28 |
| 100–150 | 15 | 21.0 | 11 | 30 |
| More than 150 | 4 | 25.6 | 21 | 32 |

longer it will require for complete recovery. Size of the larva has no influence on recovery, while those larvae which became desiccated in a shorter time also required less time for recovery. This is probably due to the fact that, in general, those larvae which required a longer time for desiccation had also lost more water. The principal factor

concerned with the time of recovery seems to be the extent of desiccation.

## DISCUSSION

One of the most obvious features disclosed by these studies was the tremendous individual variation in the rate of desiccation under approximately the same environmental conditions. Similar variations were reported by Gunn (1933) for the cockroach, *Blatta orientalis*. He found that in dry air at 30° C., one animal lost 5.5 per cent of its weight per day and lived for 8 days, while another lost 32.2 per cent and died in 1 day. This range corresponds to that reported in the present paper. Gunn states that the most careful attention to physical conditions hardly reduced the variation at all, and that during the course of a very large number of experiments the real cause of much of the variation was not discovered.

The curves of Fig. 1 show that each stage loses water more rapidly at the beginning of the desiccation period and that the rate of water loss progressively decreases. Buxton (1930), working with the meal worm, *Tenebrio molitor*, and Mellanby (1932*b*) with the bedbug, *Cimex lectularius*, also reported progressive decreases in the rate of water loss. Mellanby believes that it may be due to a decrease of metabolic rate as desiccation and starvation proceed. In the present experiments, it was noted that the activity of the larvae decreased and that they were practically quiescent by the end of the first day. It seems likely that this quiescent condition was associated with a decrease in metabolic rate and that it might result in a decrease in the rate of water loss. Other evidence for this view is the relatively slow rate of desiccation of prepupal and pupal stages. The transformation to the prepupal stage is characterized by a decrease in metabolic rate; and the metabolic rate of the early pupa is very low, being represented by the low part of the U-shaped curve characteristic of metamorphosis (Ludwig, 1931).

The maximum fatal limits of desiccation of the Japanese beetle larvae are very similar to those reported by Breitenbecher (1918) for the potato beetle, *Leptinotarsa decemlineata;* by Hall (1922) and by Buxton (1930) for the meal worm, *Tenebrio molitor;* by Speicher (1931) for the Mediterranean flour moth, *Ephestia kuehniella;* and by Buxton (1932) for the bug *Rhodnius prolixus*. This comparison is

very interesting, since the Japanese beetle lives in moist soil and is normally never exposed to drying environmental conditions. Its ability to withstand slow desiccation undoubtedly assists it in surviving those unfavorable environmental conditions resulting from periods of deficient rainfall. On the other hand, both the meal worm and the Mediterranean flour moth live in dry atmosphere, and their food is low in moisture content. They are adapted to this type of environment by the ability to conserve metabolic water, so that when 50 per cent of the body weight is lost by desiccation the normal ratio of water to dry material is maintained. The Japanese beetle is not able to conserve body water. When it becomes desiccated, the loss of weight is due to a loss of water, the percentage of body water decreasing until death results.

At the present time, no definite information is available regarding the effects of soil moisture on the distribution of Japanese beetle larvae. However, concerning the egg stage, certain observations of Fox (1934) may be significant. He found that the beetle population in heavily infested areas was greatly reduced in 1933 as compared with 1932. He suggests that this reduction may be due to the deficient rainfall during the preceding summer at the time oviposition and hatching were at a maximum.

The change in water content accompanying metamorphosis from larva to adult parallels that reported by Ludwig (1931). The readings are, in most cases, slightly higher; but the average differences are never more than 2 to 3 per cent.

The results indicate that during metamorphosis there is a considerable range in the maximum fatal limits of desiccation, when expressed in terms of weight and as percentage of original weight. However, when expressed in terms of water content, the variations are not as great, ranging from 59.1 per cent body water in the early prepupa to 65.9 per cent in the early pupa. In a form, such as the Japanese beetle, where the loss of weight is due to the loss of water, the extent of desiccation is therefore more accurately expressed in terms of water content than by percentage of original weight lost by desiccation.

It is evident from the experiments that prepupae and pupae contain more water than is necessary for normal metamorphosis. When

early prepupae were desiccated, only a partial recovery occurred before metamorphosis proceeded. Normally, dehydration occurs during this stage; but under the condition of the experiments, an actual absorption of water occurred. When early pupae were desiccated, there was no recovery of lost water; but development continued, although somewhat retarded, until the emergence of the adult. This experiment shows that, even though male pupae may lose an average of 37.0 mg., and female pupae an average of 41.7 mg. of water, they still emerge normally. These values represent about one-half of the water normally lost at the time of emergence. An average loss of more than this amount of water resulted in the inability of the adults to emerge.

The increase in the duration of the pupal stage with decrease in water content of the pupae is in agreement with the observations of many other investigators. Headlee (1917) found that the velocity of development of the eggs and pupae of the grain moth, *Sitotroga cerealella*, and the bean weevil, *Bruchus obtectus*, varied inversely with the relative humidity of the air; whereas, in the larva and in the life-cycle as a whole, it varied with humidity. Hefley (1928) stated that the length of the pupal period of the tachinid *Winthemia quadripustulata* is greatest at a humidity of 7.1 per cent and decreases as the humidity is increased up to 73.4 per cent; and Janisch (1930) showed that the eggs of *Prodenia littoralis* developed most rapidly at 90–95 per cent humidity. On the other hand, Elwyn (1917) concluded that air humidity has no influence on the velocity of development of *Drosophila ampelophila;* and Headlee (1914) concluded, as a result of his experiments on the aphid, *Toxoptera graminum*, that air humidity does not effect the development of those insects which feed on succulent food. However, in the foregoing cases the rates of development were correlated with atmospheric humidity, but the insects were not desiccated, as in the present experiments. Speicher (1931) found that in the Mediterranean flour moth, a dry atmosphere and dry food diminishes size and greatly delays pupation. Speicher's experiments were also not comparable with the present ones, since he found that as long as the larvae and pupae survived they maintained a constant ratio of body water to body weight. On the other hand, the weight lost by the Japanese beetle during desic-

cation actually consisted of a loss of water. In general, it appears that desiccation is always accompanied by a decrease in the rate of development, regardless of whether it results in any change in relative water content.

## SUMMARY

1. The rate at which different stages of the Japanese beetle become desiccated, under approximately identical environmental conditions, decreases in the following order: first,- second-, third-instar larvae, late prepupae, early prepupae, and pupae.

2. The maximum fatal limits of desiccation, expressed in weight and as percentage of original weight, varied as follows: second-instar larvae, 45 per cent; first- and third-instar larvae, 50 per cent; early prepupae, 56 per cent; late prepupae, 66 per cent; and pupae, 69 per cent. When expressed in terms of water content, the variations were greatly reduced, as shown by the following figures: third-instar larvae, 62.0 per cent; early prepupae, 59.1 per cent; late prepupae, 63.4 per cent; and pupae, 65.9 per cent.

3. The water content of the adult beetle is approximately the same (66–68 per cent) regardless of whether it had been previously desiccated in the prepupal or the pupal stages. Water content is restored by a loss of less water at the time of emergence. When larvae are desiccated, water content is restored before development can proceed.

4. The prepupal and pupal stages contain more water than is necessary for normal metamorphosis. Pupae can be desiccated until they lose about one-half of the water normally lost at the time of emergence, and still emerge normally.

5. The duration of the pupal stage is increased by desiccation of either the prepupal or pupal stages. This increase is inversely proportional to the water content of the pupae.

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# ELECTROKINETIC STUDIES OF MARINE OVA. III. THE EFFECT OF DILUTION OF SEA WATER, AND OF SODIUM AND CALCIUM UPON THE SURFACE POTENTIALS OF ARBACIA EGGS

(One figure)

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IT HAS long been known that the electrokinetic potential of a colloid is affected by various factors in the ionic composition of the medium. Among these, the concentration factor and the valency factor are of great importance; and they have repeatedly been studied by many investigators in the field of colloid chemistry (Abramson, 1934; Freundlich, 1930). The object of the present paper is to examine similar relationships, but in connection with the surface potential of marine ova.

The material used is the eggs of the sea urchin, *Arbacia punctulata*, obtained at the Marine Biological Laboratory, Woods Hole, Massachusetts, during the summer of 1934. The method of measuring the cataphoretic potential here employed is essentially the same as was described by the author in a previous paper (Dan, 1933). The zeta potential was calculated by the classical equation

$$u = \frac{DH\zeta}{4\pi\eta},$$

where $u$ is the migration velocity in centimeters per second; $\eta$, the coefficient of viscosity of the medium; $D$, the dieletric constant of the medium (here this is taken as 80); $H$, the potential gradient in electrostatic units; and $\zeta$, the zeta potential, also in electrostatic units. However, $\zeta$ will be given in millivolts.

The viscosity of various solutions was measured by a viscosimeter of the Hess type and was made comparable to that of sea water. The strength of the electroendosmotic currents in these solutions also varied widely from one case to the other. The corrections for these

differences were calculated from direct observations of the electroendosmotic currents in the solutions by using quartz particles. However, when a series of solutions was employed, each member of which differed in composition by slight gradations, the measurements were not extended to every solution of the series. In these cases, only the correction values for the more important steps were directly obtained, and the values for the intermediate steps were later calculated from a smooth curve which connects the known points.

It will also be mentioned here that, in the experiments which follow, eggs were used from which the jelly had previously been removed. Therefore, when the surface charge is mentioned, it refers to the charge on the surface of the cell and not the charge on the jelly, unless otherwise stated.

## DILUTION EXPERIMENTS

In using biological material such as sea-urchin eggs, precautions must be taken to keep the osmotic pressure as nearly normal as possible through the course of the experiment. This fact restricts the range of variation of the ionic strength of the medium a great deal. In the dilution experiment, normal sea water was diluted to 85, 70, and 55 per cent; and the potentials were measured in the respective cases. As is well known, the eggs change their volume in different concentrations. Data for the volume change were taken from a curve in the paper of McCutcheon, Lucké, and Hartline (1931); and the corrections for the electroendosmosis involved in the cataphoretic measurements of large particles (see Dan, 1933, p. 483) were calculated according to their figures.

Fifty-five per cent dilution is not less than half the original concentration; and this may be too insignificant a change in comparison with typical concentration studies, which often cover the range from zero concentration up to 1 molar or more. For this reason, it was suspected from the beginning that there would be no detectable change before and after the dilution within this range. Yet this series was studied for two reasons: First, since these concentrations have been tried in many other physiological studies, it may not be useless to run the parallel experiments in cataphoretic measurements. Second, the author suspected that an interesting find-

ing of Lucké, Hartline, and McCutcheon (1931), that the permeability constant of *Arbacia* eggs for water is different for exosmosis and endosmosis, might have some connection with the change in the electrokinetic potential, inasmuch as recent studies tend to indicate that the elasticity of the cell membrane is negligibly small (E. N. Harvey, 1931; Kamada and Yamamoto, 1931; Cole, 1932; Cole and Michaelis, 1932). The result turned out to be negative, and the potentials in these concentrations are practically identical with each other. However, it will be stressed once more that this does not necessarily mean that the concentration effect does not exist for sea-urchin eggs. It might have been detected, if sufficient range of dilution could be used. The figures are given in Table I. It is also possible to concentrate the sea water and examine the effect of it on the zeta potential. But, so far, this has not been tried.

TABLE I

THE CATAPHORETIC POTENTIALS OF THE EGGS OF *Arbacia punctulata* IN NORMAL AND DILUTED SEA WATER

The figures are given in millivolts with the standard errors

| 100 per Cent | 85 per Cent | 70 per Cent | 55 per Cent |
|---|---|---|---|
| −30.3±0.47 (1932) | −30.2±0.54 | −29.1±0.78 | −29.7±0.58 |
| −29.9±0.57 (1933) | ........ | ........ | ........ |
| −29.7±0.65 (1934) | ........ | ........ | ........ |

SODIUM-CALCIUM COMBINATIONS

For the study of the valency effect, sodium and calcium were used. As was mentioned beforehand, if it is possible to construct two curves, one for NaCl and the other for $CaCl_2$, from zero up to any desired concentrations, then by comparing the two the valency effect can be studied in a simple way. However, since the osmotic pressure of the medium should be kept constant for the present material, the following procedure is the only method available which enables us to investigate the valency effect. This is to take isotonic NaCl* as one extremity and isotonic $CaCl_2$† as the other ex-

* ½ M NaCl rendered alkaline by $NaHCO_3$. Ag-free NaCl by Merck was used.

† ⅓ M $CaCl_2$. The solution of "Baker's analyzed" preparation gives the reaction of pH *ca.* 8.1, owing to $Ca(OH)_2$ present.

tremity of the experimental conditions. For intermediate steps, both solutions were mixed in appropriate ratios. This makes it possible to change the calcium content without upsetting the isotonicity of the resulting mixtures. Then a curve can be drawn relating the potentials and calcium concentrations. But as is clear, the curve thus obtained is not comparable with the above-mentioned NaCl- or $CaCl_2$-curve, respectively. This curve corresponds to one which connects two points, one on a NaCl-curve at 1/2 M to the other on a

TABLE II

THE ZETA POTENTIALS IN VARIOUS SODIUM-CALCIUM MIXTURES AND UNDER OTHER RELATED CONDITIONS

Figures are given in millivolts with standard errors

| Solutions | Potential Difference | No. of Cells Measured |
|---|---|---|
| Sodium-calcium series: | | |
| 1/3 M $CaCl_2$ | $-13.7\pm0.57$ | 62 |
| 1/6 M $CaCl_2$ in NaCl | $-21.6\pm0.73$ | 59 |
| 1/10 M $CaCl_2$ in NaCl | $-25.0\pm0.59$ | 54 |
| 1/25 M $CaCl_2$ in NaCl | $-27.5\pm0.67$ | 55 |
| 1/175 M $CaCl_2$ in NaCl | $-30.0\pm0.61$ | 59 |
| 1/250 M $CaCl_2$ in NaCl | $-29.5\pm0.74$ | 78 |
| 1/1,000 M $CaCl_2$ in NaCl | $-25.5\pm0.53$ | 40 |
| 1/2 M NaCl | $-20.3\pm0.55$ | 50 |
| First washed in NaCl (potential difference measured in sea water) | $-19.7\pm0.54$ | 64 |
| First washed in urea (potential difference measured in sea water) | $-20.5\pm0.44$ | 69 |
| Sea water | $-29.7\pm0.65$ | 71 |
| Calcium-free sea water | $-31.0\pm0.64$ | 79 |

$CaCl_2$-curve at 1/3 M. Moreover, as the curve is followed from the NaCl end to the $CaCl_2$ end, every three ions of sodium are replaced by two ions of calcium. In spite of all these drawbacks, it should be possible to detect qualitatively the existence of the valency effect, if it occurs. The result shows clearly that this is the case.

The data are summarized in Table II, and a curve constructed from them is shown in Figure 1. (The reader's attention will more conveniently be called to this figure in a later discussion.) The ordinates in the figure represent the zeta potentials in millivolts,

and the abscissae represent the ratios of the mixtures of isotonic NaCl and isotonic $CaCl_2$, the ratios being shown as the final calcium concentrations in the resulting mixtures. The values shown in a special section are those obtained in sea water. ●'s are values observed for living cells in sodium-calcium mixtures, ▲'s are those for cytolyzed cells. ▼ is for the cells treated with urea before transferring them to sea water, and ■ is for those treated with Herbst calcium-free sea water.

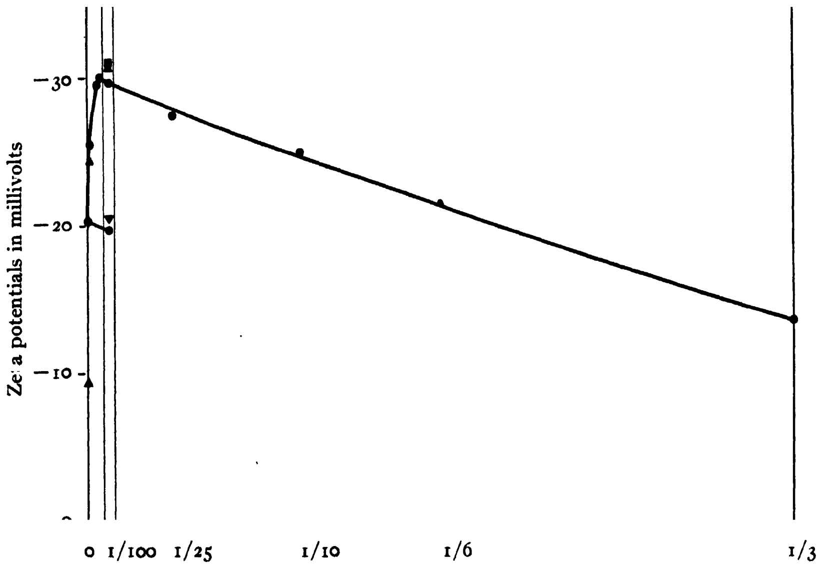


FIG. 1.—The effect of sodium and calcium ions on the zeta potentials of *Arbacia* eggs

As a starting-point, it will be convenient to choose the value in normal sea water. Strictly speaking, this point does not belong to the present series because of the Mg, $SO_4$ and other ions found in sea water besides Na, Ca and Cl. However, this figure is given because this is a convenient value for the purpose of comparison, since in some cases eggs were returned to sea water after having been in various other solutions. Moreover, this is the most reliable figure, as it has been repeatedly confirmed in the past three years. The value of the zeta potential for normal sea water will temporarily be placed on the curve in a special section at the level of 1/100 M according to

its calcium content, neglecting Mg and $SO_4$, etc. (Wheeler, 1910). At any rate, this point serves simply as a reference point.

In the beginning, the higher concentrations of calcium will be examined. As is clear from the graph, when calcium is made more and more concentrated, the absolute magnitude of the negative potential on the surface of the egg cell decreases. When finally 1/3 M $CaCl_2$ is reached, the zeta potential, which was about $-30.0$ mv. at the start, now becomes $-13.7$ mv. This decrease in the absolute magnitude of the negative potential is obviously the ionic effect due to calcium ions. However, stronger support for this explanation can be found in the following fact: If a measurement of the potential in one solution is first made and then the same eggs are transferred to another different solution, the potential can be shifted either to a higher or a lower level according to the concentration of calcium in the second solution. Values for 1/6 M and 1/25 M on the curve were thus obtained. Eggs used in both cases were derived from the same female. They were put in 1/6 M first, and after the measurement they were then washed with 1/25 M calcium. The interval between two sets of experiments was about 10 minutes, which was the minimum time required to secure a complete change of solution (four washings). This demonstrates clearly that on this side of the curve the magnitude of the potential is a function of the composition of the outer medium, and not of anything inherent in the cell membrane.

Contrary to this, the situation is very much different on the left-hand side of the curve. At 1/175 M and 1/250 M, the curve remains practically level. But when it approaches 1/1,000 M, the absolute magnitude begins to descend; and in 1/2 M NaCl it drops to $-20.3$ mv. If the valency effect is the only factor at work, the absolute magnitude of the potential ought to be the highest in 1/2 M NaCl, for there are no divalent cations in this solution. This statement is true either with inanimate particles, such as quartz, or with living cells when the surface is believed to be non-reactive with the medium (Winslow, Falk, and Caulfield, 1923; Mudd, Mudd, and Keltch, 1929). Now, if the eggs, the potential of which has been once lowered in NaCl, are returned to normal sea water, their potential does not return to the original level but stays permanently low. This makes a strong contrast to the aforementioned change in potentials

in the region of higher calcium concentration, which is characterized by its complete reversibility. This piece of evidence decisively shows that the drop in potential in NaCl is not a simple ionic proposition but must be something more inherent in the nature of the cell membrane. Or, in other words, it suggests that something irreversible has happened or else that something must have been added to or removed from the surface by washing the cells in NaCl. More detailed discussion will be given later; but it may be mentioned here that the author inclines to believe, for the present, that this is due to a permanent loss of the outermost layer.

## CYTOLYZED EGGS

In the second paper of this series (Dan, 1934), the potentials of living and dead *Asterias* eggs were compared; and it was shown that

TABLE III

THE ZETA POTENTIALS ON LIVING AND CYTOLYZED *Arbacia* EGGS IN SODIUM-CALCIUM MIXTURES AND IN SEA WATER

Potential differences are given in millivolts with standard errors

| | Normal Sea Water | NaCl Solution with 1/1,000 M Calcium-Ions | 1/2 M NaCl |
|---|---|---|---|
| Living | $-29.9\pm0.57$ | $-25.5\pm0.53$ | $-20.3\pm0.55$ |
| Cytolyzed | $-30.7\pm0.68$ | $-24.3\pm0.51$ | $-9.4\pm0.45$ |

no change could be found between the two. In the same year, a similar experiment was tried with *Arbacia* eggs, and here also the potentials on heat-killed and living *Arbacia* eggs were found to be identical. Therefore, this seems to be a general phenomenon among marine ova. During the last summer more measurements of the same sort were attempted, and the potentials in a mixture of NaCl and 1/1,000 M $CaCl_2$ and in 1/2 M NaCl alone were studied. The figures for the foregoing three cases are tabulated in Table III.

Concerning the method for obtaining cytolyzed eggs, it will be mentioned that in the case of normal sea water, eggs were subjected to 40° C. for 5 minutes. In the other two cases, because of the extremely low calcium content, the cell membranes became so weak that a slight stirring of the egg suspension caused many eggs to

cytolyze. When the eggs do cytolyze, however, the mode of cytolysis in the last two solutions is totally different. The former contains calcium ions up to 1/1,000 M. Therefore, when eggs cytolyze in this solution, they form a typical "surface precipitation membrane" in Heilbrunn's sense (Heilbrunn, 1927); while in 1/2 M NaCl, protoplasm streams out without forming any membrane. In NaCl this condition of the eggs is called "cytolysis." Therefore, strictly speaking, it is the potential of protoplasmic granules in NaCl which is measured. As will be noticed immediately, Table III shows that, as long as there are calcium ions, potentials on living and cytolyzed eggs behave correlatively, while only in the absence of calcium ions cytolyzed egg cells (or protoplasmic granules) acquire an entirely different potential fiom that of the intact cell surface.

## DISCUSSION

The measurements of cataphoretic potentials in sodium-calcium mixtures show two main facts. The first is that when calcium ions are contained in a medium beyond a certain critical value, the zeta potentials of *Arbacia* eggs behave like potentials on inanimate colloids; i.e., the absolute magnitude of the negative potential decreases when the concentration of divalent cations increases. And, also, this shift in potential is completely reversible. It can be changed at will in either direction by regulating the calcium concentration of the medium. The second fact is that below the critical value of calcium ions, the absolute magnitude of the negative potential on the egg cells again drops, but this drop is very sudden and irreversible. A close examination of these results led me to conclude that this irreversible change in potential in NaCl is caused by a permanent loss of some coating layer from the cell surface.

There are innumerable facts indicating that the cell surface reacts with calcium ions. It is quite a universal fact that the cell membrane is weakened a great deal if calcium is removed from the medium. The well-known discovery of Herbst (1900) that blastomeres of *Echinus* fall apart in calcium-free sea water indicates that the cell surface reacts with calcium, thus giving rise to a cement substance which is probably a calcium gel of some sort. Galtsoff's study on the aggregate formation from dissociated cells of *Microciona* (1925)

shows that a similar mechanism is involved in this phenomenon. Gray investigated the dissolution of the cell matrix in *Mytilus* gills (1926). He emphasized the predominant rôle played by magnesium in securing the normal condition of the tissue. But even in his case, he mentioned that calcium ions are necessary to make the complete stability of the tissue possible for more than 48 hours. Besides these, the fertilization reaction which involves a series of changes in the cortex is known to be impossible in the absence of calcium. It is also known that the hyaline plasma layer fails to appear if fertilized eggs are transferred to a calcium-free medium immediately after fertilization. Moreover, similar facts are frequently met with among plant cells. Hansteen Cranner's classical papers elucidated the problem more successfully than in any other cases (Hansteen, 1910; Hansteen Cranner, 1914); and other works with their reviews can be found in his papers. Benecke's work (1898) also led to a conclusion of the same sort.

A slightly different aspect of a similar phenomenon is approached by Heilbrunn in his study of the surface precipitation reaction. This is also a widely occurring phenomenon among different types of protoplasm (see Heilbrunn, 1928, chap. 13). More recently, Weber (1932) drew attention to an analogous behavior in *Spirogyra*. These facts, enumerated above, may not necessarily belong to a single category. Most likely they may represent diverse reactions occurring on the surface. But it suffices if they can illustrate how frequently calcium ions react with protoplasm at the boundary surface.

With these facts in mind, it is not surprising to think that there is a coating of a calcium gel on the surface of the normal *Arbacia* eggs, even though its existence cannot be detected by microscopical techniques at present. If this is admitted, it is not hard to imagine that this layer will be dissolved in case calcium is removed from the medium. Peptization of this hypothetical calcium gel in calcium-free media is again paralleled by the dissolution of calcium mucinate (Gray, 1931) and calcium caseinate (Robertson and Miyake, 1916) among inanimate substances. And of great interest is the fact that the eggs of sea urchins have two kinds of substances which actually behave in the same manner. The one is the surrounding jelly. As everybody knows, NaCl dissolves the jelly. The other is the ecto-

plasmic layer of the fertilized cells. As far as the writer is aware, E. B. Harvey (1934) was the first who noticed it. Quoting her paper (p. 232), she states:

> If the eggs after removal from the centrifuge are left in the same solution (i.e., minus Ca) or transferred to calcium-free sea water, the perivitelline space remains perfectly clear and there is no ectoplasmic layer on the surface of the eggs. If the eggs, on removal from the centrifuge, are put in normal sea water, the perivitelline space becomes filled with many small refringent spherical or oval bodies (Fig. 7), the precipitation product of the ectoplasmic material in the presence of calcium. On return to calcium-free sea water, these are again dissolved and can be precipitated again in the presence of calcium. The ectoplasmic material, after being centrifuged off as a ring, may also be dissolved in calcium-free sea water and be precipitated again as scattered spherules when returned to sea water.

My friend, Mr. T. Yanagita, quite independently noticed this reversible precipitation and dissolution of the hyaline plasma layer in the eggs of *Strongylocentrotus pulcherrimus*, and he has been comparing the potencies of various salts to cause a reprecipitation of the dissolved ectoplasmic material. He is so generous as to permit me to quote a part of his unpublished data. It is sufficient for me to mention here that Mr. Yanagita found that magnesium can also precipitate the ectoplasmic material but that the minimum concentration required for magnesium to cause the precipitation is about 60 times greater than that required for calcium.

Let us view the whole thing from this standpoint. From the foregoing illustrations it is safe to say that the cell surface of sea urchin eggs reacts with calcium. At the critical amount of calcium, the surface is just completely covered by the calcium gel. On increasing the calcium content beyond this value, the nature of the cell surface cannot be changed any further. The surface is saturated with calcium, so to speak. The thickness of the coating layer might become greater, but still its nature will remain unchanged as far as the electrokinetic properties are concerned. Then, for higher concentrations of calcium, the cell surface of *Arbacia* can be looked upon as an inert surface; and it would be expected that it ought to behave like any inert colloidal particle, that is, the zeta potential ought to be entirely dependent upon the composition of the medium. This is demonstrated clearly in our result.

Now for dilute concentrations, this gel may begin to be dissolved. At 1/1,000 M calcium, the curve is halfway down to the NaCl level. This may indicate that the surface is only partially covered by the gel, leaving the "naked" surface exposed to some extent. Finally in 1/2 M NaCl, this "naked" or underlying surface alone is being dealt with. Pushing one step forward, if this be true, NaCl may not be the only reagent which can dissolve this outer layer. Any solution devoid of calcium would serve for the same purpose. Isotonic urea was chosen for the test. In this case, the potential was not measured in urea because of its being a non-electrolyte. But the cataphoretic measurement was made after returning the eggs to sea water. As is shown on the graph, it turned out to be −20.5 mv., thus coming very close to the value of eggs treated with NaCl. The effect of sugar seems to be in the same direction, even though no quantitative study was made (see "Electrokinetic Studies of Marine Ova. IV" [this journal, p. 58]).

The nature of this outer layer of calcium gel seems to be very much the same as that of the surface precipitation membrane of Heilbrunn. As a matter of fact, it is not possible to differentiate between the two as far as the cataphoretic behavior is concerned. Probably the reason why the death of the cells does not modify the zeta potential in either *Arbacia* or *Asterias* eggs will find its explanation in the following way. At the moment the cell membrane loses its semi-permeability by death, the surface precipitation reaction is propagated throughout the cytoplasm, thus enveloping the dead cytoplasmic mass with the surface precipitation membrane, which is presumably a kind of calcium gel. Then it follows that we are dealing with the same calcium compound either in intact cells or in dead cells. Heilbrunn reported in his recent work (1934) that the inhibiting effect of anesthetics on the surface precipitation reaction can be observed in a very low concentration of calcium. He writes that this antagonism cannot be observed in normal sea water, and he ascribed it to an overwhelming abundance of calcium in sea water. This may indicate, even though indirectly, that in the low concentration he used the surface precipitation reaction is barely happening. Therefore 1/1,000 M was chosen, and the cataphoretic potentials of both normal and cytolyzed eggs were measured in this concentration. The

result is very interesting. The potentials on both normal and cytolyzed eggs decreased in their absolute magnitude, and they did it to the same extent (see Table III). This means that when the surface precipitation membrane is only partially formed, owing to a low calcium content, the outer gel layer is also partially destroyed, and the effect on both seems to be to the same degree. In other words, the decrease in the absolute magnitude of the negative potential indicates that in these cases the underlying surface has begun to show its effect (see Dummett and Bowden, 1933; Moldowskaya, 1933), and the fact that both turned out to be about $-25.0$ mv. suggests that the ratio of the area of the surface covered by the gel and that left uncovered must be about equal in both cases. Coming this far, it can easily be expected that if there is an experimental condition in which the difference between the cell surface and the internal protoplasmic granules becomes evident, it must be in some medium where this gel can be removed completely, for if a film of the gel remains, it may camouflage the underlying surface. This expectation proved to be true. As is shown in Table III and in Figure 1, in NaCl—and only in this solution—the living cell surface and the protoplasmic granules show widely different potentials.

The preceding experiments naturally led me to try the effect of Herbst calcium-free sea water. Somewhat contrary to my expectation, the value turned out to be practically the same as the normal one. Probably the explanation may be as follows: This calcium-free sea water contains a fairly high amount of magnesium, a much higher concentration than the critical value of magnesium for precipitating the ectoplasmic material in Mr. Yanagita's experiment. Thinking from the fact that the peptization of calcium mucinate is also retarded in the presence of divalent cations, it is very plausible to think that the calcium gel was protected from dissolution by magnesium ions contained in Herbst calcium-free sea water, or at least it must have required a much longer time for the gel to be dispersed than under the ordinary condition. When this series of experiments was being conducted, the time factor was not taken into consideration. Usually, eggs were left in solutions from 10 to 60 minutes before potentials were measured. It is hoped that an opportunity will be found to investigate this point more fully in the future.

Summarizing, in higher concentrations of calcium ions, either from the complete reversibility of changes in potential or from the general shape of the potential curve, it is clear that we are dealing with the ionic effect on an inert surface. On the side of greater dilutions, we notice the striking fact that potentials of *Arbacia* eggs tend to group themselves into three classes under widely diverse conditions: the first is the level of −30 mv. (intact cells, heat-killed cells, and cells in calcium-free sea water belong to this group); the second is the level of −20 mv. (eggs washed in NaCl and measured in NaCl, the same measured in sea water, and cells treated with urea and measured in sea water); and the third is the level of −10 mv. (cytolyzed cells in NaCl). This fact indicates that here we are dealing with three distinctly different surfaces, namely, the outermost gel layer, the "true" cell membrane, and the surface of the internal granules.

I acknowledge my indebtedness to Dr. L. V. Heilbrunn, whose kind suggestions and vivid discussions I found most inspiring.

## SUMMARY

1. The concentration effect and the valency effect of the medium on the zeta potentials of *Arbacia* eggs were investigated.

2. No effect of dilution could be detected within the range of dilution employed in the present study (between 100 per cent and 55 per cent sea water).

3. The study of the effect of isotonic mixtures of NaCl and $CaCl_2$ in various ratios brought out the following facts:

*a*) In higher concentrations of calcium, the surface of *Arbacia* eggs behaves as an inert surface, that is, the absolute magnitude of the negative potential decreases when the concentration of divalent cations increases; and this change is reversible.

*b*) In an isotonic solution of NaCl, the absolute magnitude of the negative potential again drops, but it does so irreversibly.

*c*) In very low concentrations of calcium, the potential shows an intermediate value between those in sea water and in NaCl.

4. The comparison of the potentials on intact and cytolyzed cells shows that they have practically the same potentials and that, when they change, they do so *pari passu*, as long as calcium is present in

the medium. Only in the absence of calcium do the two potentials differ widely from each other.

5. The facts described in 3 and 4 find a simple explanation by assuming the existence of a calcium compound on the cell surface which is dispersed in the absence of calcium ions.

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# ELECTROKINETIC STUDIES OF MARINE OVA. IV. THE SURFACE POTENTIALS OF CENTRIFUGED FRAGMENTS OF ARBACIA EGGS

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WE ARE familiar with numerous experiments in which centrifugal force was applied to cells of various types. If we restrict ourselves to egg cells for the present, we find that practically all the classical papers seem to agree in two main points: first, the internal granules can easily be shifted in position; second, the cortical layer, on the other hand, is not so easily affected (at least by hand centrifuges).

More recently, however, owing to technical improvements, it has become relatively easy to employ much stronger forces. As a result of this advancement, evidences now seem to be accumulating that the cortical material also suffers a shifting if much stronger forces are applied on it.

Lindahl (1932), in his dark-field examinations of centrifuged eggs of *Arbacia pustulosa*, recognized three layers on the surface of unfertilized eggs. These are the outer shining layer, the middle dark layer, and the innermost shining layer. This innermost layer is the lipoid layer, according to Runnström (1923). In his extensive studies (1928), Runnström claimed that *Arbacia* was exceptional in lacking this shining layer. However, Lindahl, after confirming the former's description in uncentrifuged eggs, discovered that this layer is detectable after centrifuging. As is clear from Lindahl's figures, the two inner layers thin out toward the centripetal pole and thicken toward the opposite end. At any rate, it is beyond doubt that the cortical material changes its normal distribution after centrifuging.

A more spectacular case of a similar kind was reported by E. B. Harvey (1934). She found that the ectoplasmic layer of fertilized sea-urchin eggs can be thrown off the eggs completely in a ring (*Parechinus microtuberculatus*). This fact indicates that the cortical

layer is composed of a heavy material which usually maintains its integrity by its high viscosity, but can eventually be thrown down if a strong enough force is applied.

A more important phase of this line of study, however, may be the fact that the membrane-forming capacity of centrifuged eggs seems to be closely correlated with the distribution of their cortical material.

Taylor first reported (1931) that the centrifuged eggs of *Urechis caupo*, on insemination, form more or less triangular fertilization membranes. This is due to the fact that the membrane elevation is very slight at the centripetal end, while it is more conspicuous on the opposite side. Thus the membrane broadens toward the heavy pole. A series of beautiful photographs of Costello's experiments (1935) on starfish eggs speaks for this most clearly and most emphatically. In this case, not only the perivitelline space becomes extremely narrow on the centripetal end but the membrane is missing entirely at this pole. Therefore, in lighter halves, after several cycles of cleavage, blastomeres escape through the opening in the membrane, and they are scattered in the medium.

As to the sea-urchin eggs, Harvey states:

> If broken apart (in tubes at 11,000 times gravity) before the elevation of the fertilization membrane, this membrane may subsequently form over each part, but is thinner (ruptures more easily) over the centripetal pole of the part; it is also thinner over the oil cap of the elongate whole egg [Harvey, 1933*a*, p. 389].

Now, with these facts in mind, a series of experiments was undertaken to see whether or not a change in the electrokinetic potential of the eggs of *Arbacia* accompanies the fragmentation by centrifuging.

## MATERIAL AND METHOD

The experiments, the results of which are to be presented in this paper, were performed during the summers of 1933 and 1934 at the Marine Biological Laboratory, Woods Hole, Massachusetts. The eggs of *Arbacia punctulata* were used as material. They were obtained by making ripe females shed by removing the oral parts. The fragmentation by centrifugal force is effected according to Harvey's method, that is, by stratifying the egg suspension over isotonic sucrose solution in centrifuge tubes. After the separation of lighter and

heavier fragments, they were washed several times in sea water. The cataphoretic potential was measured by the method previously described (Dan, 1933). A correction for the electroendosmotic flow, which was also discussed before, eliminates automatically a possible influence of the size difference between lighter and heavier halves on the apparent speed of cataphoretic migration.

When the separation is completed, the jelly is usually thrown off entirely. Therefore, no special treatment was employed for this purpose. However, in order to secure the complete removal of the jelly, the egg suspension was sucked in and out of a pipette during the washing process. The complete removal of the jelly can also be checked by the careful examination of the speed of fall of these fragments at the time of the potential measurements, for eggs with the jelly fall considerably more slowly than those without it. Thus it will be understood that the potentials given below refer to those of eggs or egg fragments with the jelly removed.

## RESULTS

In the summer of 1933, egg suspensions (in sea water) were stratified over isotonic sugar solutions in order to get separation into fragments. The potentials on the fragments thus obtained are given in the first row of Table I. As will be seen, the potential on the centrifuged non-fragmented eggs was $-26.0$ mv.; on the heavy halves, $-25.6$ mv.; and on the light halves, $-21.2$ mv. At that time, the author could not give any plausible explanation for these results. However, toward the end of the season, it began to be suspected that the use of isotonic sugar solution itself seemed to modify the potentials of the eggs; and, moreover, various indirect evidences suggested that this was apparently due to the absence of calcium in the sugar solution. Therefore, in the following summer, a similar experiment was repeated, but the isotonic sugar solution was replaced with an isotonic mixture of sugar and $CaCl_2$. The calcium concentration was so adjusted as to be about the same as that occurring in the natural sea water. This causes a prolongation of the time of centrifuging necessary for fragmentation. The potential measurements also showed a slightly different result. The figures are given in the second row of the table. Here the potential on the centrifuged non-frag-

mented eggs came out as −30.8 mv., a more negative potential. As was shown in previous papers, this value is identical with the potential on the normal eggs if the jelly is removed from them. The heavier halves also acquired a slightly more negative potential, and the value for the lighter halves remained practically unchanged.

The writer thinks that in the light of the conception arrived at in the previous paper of this series ("Electrokinetic Studies of Marine Ova. III" [this journal, p. 43]), we are now in a position to give an explanation for the foregoing results. Summarizing the conclusion reached at the end of the previous paper, it is roughly as follows: (1) The surface of the egg cell (jelly removed) is covered by a coating

TABLE I

CATAPHORETIC POTENTIALS OF EGG FRAGMENTS

Figures are given in millivolts with standard errors

| | Centrifuged Non-fragmented Eggs | Heavy Fragment | Light Fragment |
|---|---|---|---|
| Experiment performed in 1933 (isotonic sugar was employed)....... | −26.0 | −25.6±0.84 | −21.2±0.64 |
| Experiment performed in 1934 (isotonic mixture of sugar and $CaCl_2$ was used)..................... | −30.8±0.54 | −27.6±0.35 | −20.9±0.69 |

of a calcium compound. This layer is characterized by a potential of −30 mv. (2) This layer is dispersed in media lacking in calcium. Then an underlying surface becomes exposed. This new layer is characterized by a potential of about −20 mv. (3) When the calcium concentration is extremely low, the potential falls to somewhere between −30 and −20 mv., owing presumably to a partial dispersion of the calcium compound described above.

Now, it is extremely interesting to find that all six values given in the table fall in one of the foregoing three classes. Beginning with the case of centrifuged non-fragmented eggs, when calcium ions are involved uniformly in two stratifying solutions, the potentials on these eggs coincide with the value of the normal uncentrifuged eggs (−30 mv.). But if the addition of calcium to the sugar solution is omitted, the potential of the centrifuged non-fragmented eggs drops

to $-25$ mv. This potential happened to be the same as was obtained in a mixture of NaCl and 1/1,000 M $CaCl_2$ in the previous paper. Now, when the egg suspension in sea water is stratified over the isotonic sugar solution and centrifuged, a minute amount of calcium from the sea water must, no doubt, have been mixed with the sugar. There are several reasons to believe so.[1] And if this is really the case, we have to expect that this group should acquire a potential characteristic of those eggs which were treated with a medium containing a trace of calcium.

On the contrary, the potentials on the lighter fragments stayed at the level of $-20$ mv. in both sets of experiments. This fact shows that on the surface of the light fragment, the calcium gel is absent. If so, it naturally follows that the absence or the presence of calcium ions cannot cause any change in potentials, as there is no calcium gel to be affected.

Assuming that the foregoing explanation is correct, this result gives us some insight to the change occurring on the surface at the time of fragmentation. That is: as long as the cells remain intact, the calcium layer seems to cover the entire surface of the cell even after a long centrifuging, for if the layer is partially thrown down, leaving the underlying surface exposed, the absolute magnitude of the resulting potential ought to be less than 30. When, however, the separation has taken place, the lighter halves acquire a potential of $-20$ mv. This means that on the surface of the lighter halves, the calcium gel is missing. Then the question arises as to where the calcium gel has gone. The present results suggest that the calcium

[1] There are three reasons: (1) When the stratification was made, it was my usual practice to fill the centrifuge tube first with the suspension; then the sugar solution was introduced to the bottom of the tube by a long pipette. Therefore, when the sugar was poured out of the pipette, it pushed the lighter suspension up from below. As a result, it is evident that a small quantity of sea water must have been adhering to the surface of the tube. (2) After the stratification was made, the boundary of the upper sea water and the lower isotonic sugar was intentionally stirred in order to get a gradual transition between the two solutions. This was done because the less steep the gradient of specific gravity change, the more efficient it was to cause the separation of two halves. (3) When the eggs were fragmented, the heavier halves sank to the bottom of the tube through the sugar solution. Therefore, when a great number of the eggs were fragmented at the same time, an appreciable amount of calcium must have been carried with them into the sugar solution.

layer is thrown down to the centrifugal pole of the heavy halves. There are two reasons to support this view:

1. As can be learned from Table I, in the experiments performed in 1934 the heavier halves have a potential of $-27$ mv. If the calcium layer remains and covers the whole surface area of the heavy halves, the potential must be $-30$ mv.; while, if this layer is completely cast off, the potential must be $-20$ mv. The fact that this potential lies between $-30$ and $-20$ may indicate that on the surface of the heavier halves both kinds of surfaces are being exposed. If we follow this simplified picture a step farther, it means that one third of the surface is made of the underlying layer; and two thirds, of the calcium layer according to the following simple calculation:

$$21 \times X + 31 \times (1 - X) = 27 \qquad \therefore\ X = 0.3\ .$$

2. Occasionally, eggs were found which broke up into three parts. They are the light fragment which is almost exactly like the ordinary light halves; the middle one, which takes up the most part of the ordinary heavier halves; and the heaviest fragment, which is very small and closely packed with the pigment granules. Harvey (1933*b*) noticed that the sizes of the fragments vary when the centrifugal force employed is changed. And in some of her experiments, she did get "triple eggs." However, the size relationship seems to be very much different in her experiment and in mine, and I obtained triple fragmentation only very seldom. Because of the scarcity of cases, no quantitative data could be taken. But it is beyond any doubt that the middle fragment carries the same potential as the light ones and the tiny heaviest part moves as fast as non-fragmented eggs, which shows that the potential on the heaviest fraction is close to $-30$ mv. This indicates that, when an egg is separated into three parts by the centrifugal force, the calcium layer is thrown down to the tiny heaviest part and stays there.

## DISCUSSION

If the foregoing result is looked at from a different angle, we can interpret it as indicating that, when fragmentation of the cell occurs, the outer calcium gel is thrown to the centrifugal end, and that, if the fragmentation of the cell fails to happen, the sedimentation of

the outer layer also fails to occur. Now we face the question as to which one of the two is the cause and which one is the effect. At the present stage of our knowledge it is too premature to decide this point. The only fact available now is the fact that the absence of calcium in the medium shortens appreciably the time necessary for fragmentation. The writer has been following the line of thought that calcium ions solidify the outer layer, which is apparently a calcium gel of some sort. Then as far as the preceding piece of evidence is concerned, it adds some weight to the idea that it is this outer calcium layer which resists the tearing force and protects the eggs from fragmenting. Once it collapses, it snaps and shrinks to the centrifugal end, and the cell body is separated into two or three parts, as the case may be.

In the introductory part of this paper, I collected some facts indicating that in the centrifuged eggs there is a gradient of the membrane-forming capacity from the upper pole to the lower pole. As will be noticed immediately, this gradual transition in the membrane-forming capacity does not harmonize with the idea of a sudden collapse of the outer layer. I think these two things are mutually independent. The membrane-forming capacity is correlated with "the cortical layer" inside of the cell membrane. This is the cortical layer which Chambers (1921) and Just (1923) showed to be indispensable for the formation of the fertilization membrane. Independently of this, and on the outer side of the cell membrane, the calcium layer slides toward the heavy pole and the intervening cell membrane itself remains always intact. This must be the membrane which carries $-20$ mv., for, either after fragmentation by centrifuging or after the treatment with NaCl, we always find a surface characterized by the potential of $-20$ mv. spreading itself over the cell.

## SUMMARY

1. The surface potentials of the centrifugal fragments of *Arbacia* eggs were studied.

2. The lighter halves have a potential of $-20.9 \pm 0.69$ mv. and the heavier halves $-27.6 \pm 0.35$ mv., while the centrifuged non-fragmented eggs have $-30.8 \pm 0.54$ mv.

3. The results of the potential measurements indicate that the

rupture of the outermost layer (a calcium compound) occurs at the time of fragmentation.

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# MOTILITY FACTORS IN MASS PHYSIOLOGY: LOCOMOTOR ACTIVITY OF FISHES UNDER CONDITIONS OF ISOLATION, HOMOTYPIC GROUPING, AND HETEROTYPIC GROUPING

(One figure)

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SOME of the most interesting of the phenomena exhibited by animal aggregations are the peculiar forms of physiological response of a group (or of a member of a group) of organisms in contradistinction to the characteristic response of an isolated individual of the same species to certain diverse physical or chemical agencies. The "group effect" may be displayed in one or more of the following ways: by differential growth rates (Adolph, 1931; Shaw, 1932), percentages of survival under unfavorable conditions (Allee and Bowen, 1932), reproductive rates (Park, 1932, 1933), differential sex ratios (Whitney, 1929; Brown and Banta, 1935), autotomy (Allee, 1927), conditioned learning times (Welty, 1934), oxygen consumption rates (Allee, 1927; Schuett, 1933), and other like quantitative criteria. These relationships existing in animal aggregations either in nature or in the confines of experimental media have been designated as phenomena of mass physiology (Allee, 1934).

In the case of oxygen utilization, Schuett (1933) reported experiments wherein the average oxygen consumption of each fish in a group of four goldfish placed in a given volume of water was less than the oxygen utilized by one fish confined to the same volume of water. Although he arrived at no definite conclusions concerning the reasons for the "group effect" in oxygen consumption, he was able to show by suitable running-water experiments that the decrease in oxygen tension of the medium was probably not responsible for the difference in oxygen consumption and that it was improbable that an

[1] We wish to acknowledge our indebtedness to Professors F. M. Baldwin and C. C. Lindegren for facilities and valuable suggestions.

accumulation of carbon dioxide or other metabolites played an important rôle in differential oxygen consumption. In a more recent paper Schuett (1934) states that the Winkler method for determining oxygen tension as used in his previous work has been found to be at fault and that in the light of new experiments no statistical significance can be ascribed to the small difference in oxygen consumption of grouped and isolated goldfishes as reported in 1933. By the use of quantitative methods Schuett (1934) found that less locomotor activity occurred in grouped goldfish than in isolated ones. It is thus reasonable to expect a real, even though small, difference in oxygen utilization in favor of isolated individual fishes unless some other, variable factor is present to neutralize the higher oxygen requirements entailed by their increased activity.

## APPARATUS AND METHODS

For estimating the locomotor activity of fish we have devised two alternate methods, cinephotographic and observational, both depending upon the principle of plotting the spatial trajectory of the animal on three axes as a curve of closely successive points. This is made possible in both methods by optically subdividing the medium into small cubes which serve to locate the successive positions of the individual at very short intervals of time. An early version of this method has already been employed and described, with our consent, by Schuett (1934) in his studies on activity. An aquarium may be calibrated by painting parallel lines at suitable intervals on the walls of a rectangular glass aquarium, subdividing the surfaces into wide transparent stripes as follows: vertical stripes on the front wall for measuring movement on the length axis, horizontal stripes on the back wall for measuring movement on the depth axis, and vertical stripes on one end wall for measuring movement on the width axis. In order that all three sets of stripes may be viewed from the front and thus be photographed upon a single film when the cinematographic method is used, a plane mirror is placed near that end of the aquarium which lies opposite the calibrated end. Within a volume thus optically subdivided any given cube is in contact through its surfaces, edges, and corners with twenty-six adjoining cubes. There are, however, only three possible center-to-center dis-

tances between any cube and the contiguous ones. For example, in the case of cubes with a 2-cm. side, these distances are: 2 cm. for the six cubes in contact at the surfaces, 2.28 cm. for the cubes in contact at the twelve edges, and 3.46 cm. for the cubes in contact at the eight corners. Since the photographic method at once reveals the distance category of the cube into which the fish passes from an adjoining one, the total length of the trajectory is the sum of the distances between the cubes traversed. The error which arises from the fact that a fish does not proceed exactly from the center of one cube to the center of the next can be made negligible by diminishing the size of the cubes and employing as a reference point a small but distinct anatomical landmark on the fish. For example, the tip of the dorsal fin of the goldfish remains visible from all angles when 2-cm. stripes are used.

For the observational method the aquarium is calibrated as previously described. Three observers station themselves so that each observes the fish against one of the three striped walls of the aquarium, i.e., along one particular spatial axis. Each observer registers a unit on a hand tally when the fish passes one of the lines on the axis under his surveillance. The total number of stripes passed on the three axes is directly proportional to the extent of movement: the product of the width of the stripes and the number of lines passed approximates the true length of the trajectory. For accurate determinations a correction factor must be employed, since there is an inherent overestimation of the true length of the trajectory. The overestimation depends upon the shape of the trajectory and the width of the stripe used. The latter factor can be made small if the narrowest stripes allowing clear vision for the observers are used. Variations in the shape of trajectory may be corrected for as follows: A wire of known length bent into the shape of any hypothetical trajectory is placed within the calibrated aquarium. Then the length of wire is estimated by multiplying the width of the stripe by the total number of stripes that are seen to be "traversed" by the wire, as observed against the striped sides. When the percentage of overestimation ($x$) is found, a second wire, shorter by $x$ per cent than the true length of the first, is bent into another hypothetical trajectory, placed in the aquarium, and estimated for length. The mean percentage of

overestimation obtained from several such attempts may be taken as the correction factor. For stripes 2 cm. wide, as used in the experiments reported here, the correction factor is approximately 1.4 per cent.[2]

For our experiments a rectangular glass jar 24 cm. in length, 12 cm. in width, and 24 cm. in depth was calibrated with stripes 2 cm. wide and used as an aquarium, being filled with water (from a deep artesian well) to 1,625 cc., 3,250 cc., 4,875 cc., and 6,500 cc. Isolated, and in groups of four, the activity of small specimens (6–8 cm.) of *Carassius auratus* L. was studied by daylight in a room maintained at 19.5°–20.5° C. Using the observational method, several 20-minute periods of measurement were carried out on successive days until twenty observations had been recorded for both conditions of aggregation in each of the four volumes of water. We have made similar but less extensive observations on the movement of 6–8 cm. specimens of *Carassius auratus* L., *Gambius affinis* L., *Oryzias latipes* Bleeker, and *Macropodus verdi-auratus* Lacapede, under conditions of isolation, homotypic groups of four, and heterotypic groups of four in which each fish was of a different species. The four species are those mentioned above. In the heterotypic groupings, care was taken to select individuals of approximately the same size. All animals, when not under observation, were maintained in aquaria in the laboratory in which the experiments took place. Four to 8 hours were allowed for the fish to recover from the effects of handling before the actual observations on activity were begun. At all times the observers followed the movements of the fishes from behind gauze curtains to preclude any reactions that the fishes might display due to motions on the part of the experimenters. The behavior of the fishes at no time indicated their detection of the presence of the observers.

## DATA

Our experiments with *Carassius auratus* show that there is decidedly less locomotion when the fish under observation is a member of a homotypic group of four than when it alone occupies the same volume of water. This relationship holds good in each volume which

[2] The data given below are uncorrected.

we have tried, not only for mean values of the series of twenty observations (Table I), but also for most of the separate observations. Although there are within each series rather great variations from the mean, there are few overlapping values when data for the activity of isolated and grouped fishes are compared. The combined data for isolated and homotypic groups of *C. auratus* from Tables I and II are shown in Figure 1.

TABLE I

LOCOMOTION: MEAN VALUES OF TWENTY SEPARATE OBSERVATIONS IN EACH VOLUME AND AGGREGATION

*Carassius auratus*

| Volume of Water (in Cc.) | Fish A | | Fish B | |
|---|---|---|---|---|
| | Isolated (Meters per Hr.) | Grouped (Meters per Hr.) | Isolated (Meters per Hr.) | Grouped (Meters per Hr.) |
| 1,625 | 83.40 | 17.26 | 103.40 | 39.10 |
| 3,250 | 83.44 | 55.47 | 69.43 | 39.65 |
| 4,875 | 51.33 | 32.54 | 61.95 | 13.08 |
| 6,500 | 118.18 | 58.43 | 100.09 | 50.94 |

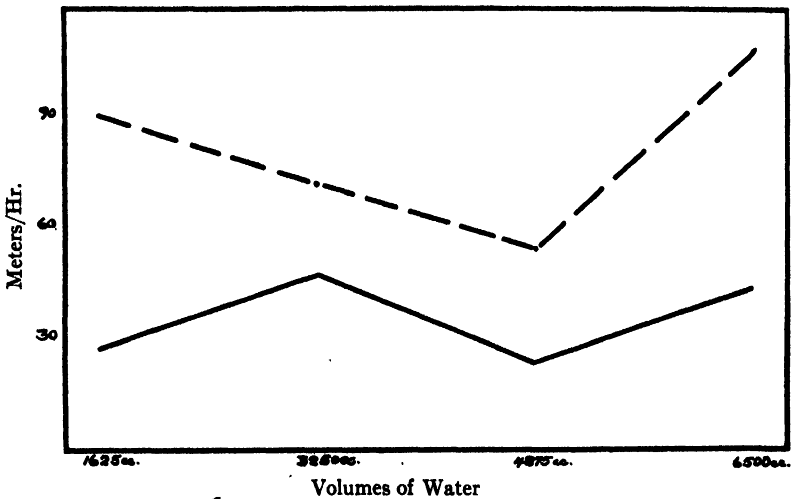


FIG. 1.—Dotted line represents movement of isolated *Carassius auratus;* solid line represents movement when in homotypic group of four. Mean values for forty-seven experiments each volume and condition of aggregation.

The activity of a solitary goldfish and of the same individual as a member of an homotypic group and as a member of an heterotypic group is summarized in Table II. These data suggest that, at least

TABLE II

LOCOMOTION: MEAN VALUES OF SEVEN OBSERVATIONS IN EACH VOLUME AND AGGREGATION

| Volume of Water (in Cc.) | Isolated (Meters per Hr.) | Homotypic Group (4) (Meters per Hr.) | Heterotypic Group (4) (Meters per Hr.) |
|---|---|---|---|
| | *Carassius auratus* (Fish C) | | |
| 1,625 | 81.06 | 24.15 | 54.21 |
| 3,250 | 59.58 | 51.84 | 110.94 |
| 4,875 | 17.88 | 31.14 | 71.88 |
| 6,500 | 108.69 | 17.58 | 126.00 |
| | *Gambius affinis* | | |
| 1,625 | 26.94 | 25.80 | 57.48 |
| 3,250 | 77.58 | 70.68 | 66.96 |
| 4,875 | 101.16 | 90.33 | 111.98 |
| 6,500 | 123.24 | 106.56 | 105.96 |
| | *Oryzias latipes* | | |
| 1,625 | 27.96 | 39.54 | 75.84 |
| 3,250 | 75.30 | 82.11 | 97.08 |
| 4,875 | 65.64 | 51.54 | 80.40 |
| 6,500 | 111.98 | 104.34 | 105.84 |
| | *Macropodus verdi-auratus* | | |
| 1,625 | 24.60 | 30.96 | 27.52 |
| 3,250 | 34.46 | 56.34 | 51.00 |
| 4,875 | 42.16 | 45.60 | 41.54 |
| 6,500 | 97.86 | 45.36 | 74.37 |

in the early stages of the artificial formation of such an heterotypic group, the several members display much more locomotor activity than in a comparable homotypic group, and slightly surpass the activity typical of the isolated individual. This observation, if subse-

quent confirmation should establish it as a constant phenomenon, might permit interpretations of sociogenic significance.

Of the other fishes studied (Table II), the activity of *Gambius affinis* most nearly ran the course described for *Carassius auratus*. The activity of *Oryzias latipes* and *Macropodus verdi-auratus*, particularly in regard to the comparatively small amounts by which the individual fishes in the heterotypic groupings excelled their own activity when in the homotypic groupings, might indicate variations in the response of the separate species to whatever integrative or dispersive stimuli exist in the respective aggregations. It is not beyond question, however, that such aberrations from the type of motility response occurring in *C. auratus* could arise from the differential effects which the temperature of the medium might exert on the various species.

Our data for the locomotor activity of isolated and homotypically grouped *Carassius auratus* and *Gambius affinis* are in agreement with the observations of Schuett (1934), who reported greater activity for isolated *C. auratus* than for groups of four in experimental volumes of 7,500 cc. and 15,000 cc. Although the plan of our experiments was such that the data derived from them do not lend themselves very well to testing Schuett's (1934) assertion that there exists an "optimal number" of individuals per unit volume of medium (i.e., that activity attains a minimum when a certain number of individuals are present), some comparison can be made between his results and ours. By far the greatest number of our observations (Table I and Fig. 1) show that minimal activity for both isolated and grouped goldfishes occurred at the same volume, namely, 4,875 cc. To this extent our experiments are not indicative of a relationship between "optimal number" of individuals and minimal activity for a given volume. Since we have dealt only with solitary fishes and groups of four, while Schuett studied, besides isolated individuals, groups of four, eight, and sixteen individuals, we cannot challenge directly his contention that an optimal number per unit volume holds wherein least activity occurs.

We were led to suspect that the spatial factors affecting total movement in different masses of water were of a more subtle nature than merely volume relationshops (i.e., total volume or volumes per

individual as expressed by previous workers). Statistical analyses of the data of 360 separate experiments show that the relative movement of fishes along the different axes of the aquarium (i.e., the particular length, width, and depth) bears a relationship to the magnitude of each of the three dimensions of the mass of liquid and only remotely to its volume. Combining the data for the goldfishes A and B in Table I, we have calculated "product-moment" correlations to show the relation between the total number of stripes traversed and

TABLE III

RELATION OF TOTAL MOVEMENT TO MOVEMENT ON ENVIRONMENTAL AXES*

| Depth (in Cm.) | *rtl* | P.E.*r* | *rtw* | P.E.*r* | *rtd* | P.E.*r* |
|---|---|---|---|---|---|---|
| | ISOLATED *Carassius auratus* | | | | | |
| 5.64 | +.995 | .0107 | −.213 | .1030 | +.437 | .0875 |
| 11.28 | +.816 | .0360 | +.168 | .1050 | +.212 | .1031 |
| 16.92 | +.232 | .1021 | +.195 | .1040 | +.416 | .0893 |
| 22.56 | +.175 | .1049 | +.381 | .0923 | +.700 | .0551 |
| | GROUPED *Carassius auratus* | | | | | |
| 5.64 | +.820 | .0354 | +.729 | .0511 | +.787 | .0395 |
| 11.28 | +.765 | .0463 | −.219 | .1028 | +.802 | .0385 |
| 16.92 | +.661 | .0609 | +.497 | .0813 | +.921 | .0185 |
| 22.56 | +.620 | .0665 | +.576 | .0721 | +.696 | .0556 |

* *rtl* = relation of movement on length axis to total movement. *rtw* = relation of movement on width axis to total movement. *rtd* = relation of movement on depth axis to total movement. 1.00 denotes complete relation. $P.E._r$ = Probable error calculated from $[0.6745 \times (1-r^2)]/\sqrt{N}$.

the number of stripes traversed on each axis. These data are given in summary in Table III.

In the case of the isolated fishes, it will be noted that the relation of movement on the length axis to total movement (*rtl*) is highest when the depth axis is most restricted, and that it diminishes as the depth increases. Since 1.00 denotes perfect relation, the correlation +.995 indicates that a very great portion of the total locomotion recorded in each experiment occurred on the length axis. A correlation below +.900 indicates a moderate relationship; less than +.700 de-

notes more or less random relationships. The values of *td* tend to increase in the same order as the increase of the depth axis, indicating that this dimension has become available for movement. The data for the relation of locomotion on the width axis to total locomotion (*rtw*) show that random movements prevail on this axis throughout.

For the grouped fishes, our data reveal no correspondingly high correlations under similar conditions; nor do movements seem to be at random to so great an extent. A member of a group does not tend to move along any particular axis so consistently. Whatever may be the influence of the magnitude of any dimension, it is apparently less powerful in the group. If one should arrange a container of such dimensions that locomotion would be unrestricted only on one dimension, one would expect a high relation between total movement and locomotion on the unrestricted axis. A fish 6 cm. in length, such as used in our experiments, placed in an aquarium 500 cm. long, 4 cm. wide, and 4 cm. deep, would be limited to movements along the length axis and might indeed experience some difficulty in reversing its course. On the other hand, little difference in degree of influence might be expected if the aquarium were a cube with a 20-cm. side, having the same volume as the former.

Furthermore, if the 500-cm.-long container described above were stood on end so that its long axis were vertical, a fish placed in it would have at its disposal a depth of 500 cm. of water for movement, without being able to move freely along the dimensions of width and length of the container. These extreme examples of spatial restriction serve to emphasize the influence that magnitude of environmental dimensions can exert on the motility of fishes.

In the previously described vessel one would expect an inclosed fish to move more freely, and hence possibly more per unit time, with the container having its long axis in the horizontal position rather than in the vertical. A priori, such behavior is expected in view of the fact that the locomotor mechanisms of fishes (with a few exceptions, e.g., seahorse) are adapted to propel the fish along the long axis of its body, the latter being normally oriented in most species of fishes in a horizontal plane. In experiments on fishes, the shape of the aquaria is usually of the form of a rectangular parallel-

opiped, in which no one dimension differs greatly from the other two. In an aquarium of this shape the horizontal components of the movement of a fish usually exceed the simultaneous vertical components of that movement.

We are inclined to believe that when a fish is limited to a given volume of water the configuration of that volume, or the interrelation between the relative magnitudes of its three dimensions, determines to a considerable extent the total amount of movement.

In view of the preceding considerations an explanation may be advanced for our finding that minimal activity in both grouped and isolated goldfishes occurred at a volume of 4,875 cc. having a depth of 16.92 cm., a width of 12 cm., and a length of 24 cm. The latter two dimensions are constant for the four volumes of water used, since they are the dimensions of the container, the depth axis alone varying with increasing volume. This is shown in Table III.

The fact of the occurrence of least locomotor activity for grouped, as well as for isolated, fishes at a volume of 4,875 cc. may possibly be attributed to the coincidence that at this particular volume and for the size of fishes used there occurred a relation between the dimensions which makes for diminished activity. It appears that the spatial characteristics of the medium other than volume influence the activity of fishes. The several axes or dimensions of the mass of water, and possibly its configuration as well, exert effects. So many variables impinge upon analyses of animal movement in tridimensional space, especially when complicated with the volume available for movement, that at present one can merely recognize certain physical and biological characteristics of the organism which are likely to interplay with the spatial characteristics of the medium to yield a specific quantity of movement.

Degree and type of somatic axiation, degree of cephalization, normal orientation in space, methods of propulsion, specific gravity, susceptibilities to geotropism, to thigmotropism, and degree of irritability to repeated contacts, etc., are biological peculiarities which probably influence the kind and extent of movement performed in restricted masses of water of diverse configuration.

The possibility that certain spatial factors other than volume may influence the behavior of active animals restricted to small vol-

umes of media is suggested by the experiments of Bilski (1921), who found that the growth of tadpoles was affected by the relative size of the vessel, the volume per individual being kept constant. Bilski believed that growth was retarded by the stimulation from contact or near approach of two individuals and that possibly such contacts were in proportion to the sizes of containers. Thus for two containers, $a$ and $b$, having an equal density of population, the probabilities of intragroup stimulation would be $a(a-1)$ and $b(b-1)$. If the relation of the size of the containers is taken as 2:3, the calculated probabilities of stimulation are 2:6.

Why an isolated fish moves more than a member of a group (at least in small containers) is a still more obscure phenomenon. After having examined motion-picture films of both isolated and grouped fishes, it is our belief that two types of behavior displayed may yield a clue to possible explanation. First, it appears that the "free path" or direction of movement of a grouped fish is being continuously obstructed by the nearby presence of his fellows. Our records show that a solitary fish describes a path or trajectory that is more smooth and much more free from reversals than the path of a member of the group. Furthermore, cinephotographic records show that members of a group are continuously making small adjustments in their orientation or movements as they react to the presence of their nearby fellows. From the examination of our motion-picture films it appears that the total movement performed during a given time interval is always less when the separate movements are short and interrupted by pauses than when they are more continuously prolonged as in the case of the solitary fishes.

Of these reactions, just what is cause and what effect remains to be determined.

It becomes increasingly obvious that, in order to obtain comparable data, investigators dealing with problems of locomotor activity must adhere to strictly controlled experimental conditions. The necessity of standard temperature, reaction, and chemical composition of media is widely recognized. It would seem justifiable to expect equal exactitude in the selection of individuals of known age and size, inasmuch as locomotor ability is closely related to chronological changes in corporal development. Particularly in experiments with

homotypic groups, the sex of adult members becomes significant in determining the behavior of the individual. Differential locomotor activity in the sexes has recently been demonstrated by Slonaker (1935) in the rat. Even in immature individuals sex cannot be ignored, since there may exist dimorphisms and differential physiological abilities. The results of investigations upon activity differences between the two homosexual homotypic groups of animals are certain to prove both interesting and sociologically significant.

In the case of animals with highly developed visual sense organs, the contour and coloration of their fellows in homotypic or heterotypic groups may conceivably alter their movements, resulting in various degrees of aggregation or dispersal.

Especially in view of the fact that the integration of certain aggregations of fishes has been demonstrated by Bowen (1931) to be visual, it becomes imperative to carry all observations on the activity of fishes under conditions of symmetrical and constant illumination. Only in this way will it be possible for even the same investigators to obtain reproducible results. Furthermore, certain experiments performed in this laboratory (unpublished) impress upon us the absolute necessity of a constant artificial illumination.

The very nature of the phenomenon of animal movement, bristling as it does with intangible and uncontrollable variables, requires from the early investigator a most scrupulous study entailing a thorough control of all the recognized factors involved.

## SUMMARY

1. Cinephotographic and observational methods for estimating the locomotor activity of animals in tridimensional space have been described.

2. In *Carassius auratus*, the activity of an isolated individual is greater than the activity of the same individual when grouped with three other goldfishes. The activity of the goldfish when as a member of an heterotypic group exceeds even that in the isolated condition. The behavior of *Gambius affinis*, *Oryzias latipes*, and *Macropodus verdi-auratus* in isolated and aggregated conditions is reported.

3. Certain suggestions concerning the selection, conditioning of experimental animals, and the control of the environment in activity studies have been made.

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# THE DIFFERENTIAL REACTION OF THE BLOOD VESSELS OF A BRANCHIAL ARCH OF AMBLYSTOMA TIGRINUM (COLORADO AXOLOTL) I. THE REACTION TO ADRENALIN, OXYGEN, AND CARBON DIOXIDE[1]

(Three figures)

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DURING a study of the problem of gill reduction in amphibians, the hypothesis that the process of gill reduction is closely related to the reactions of the branchial vessels suggested itself. A study of the reactions of the branchial vessels in *Amblystoma tigrinum* larvae was attempted, therefore, in the hope that this would either confirm or disprove the foregoing hypothesis. In addition, it was thought that such a study might add a few facts to our knowledge of the general physiology of blood vessels.

The present paper will deal with the reaction of the branchial vessels to adrenalin, oxygen, and carbon dioxide. Evidence will be presented to show that there are two sets of blood vessels in the same gill arch, one set of which dilates under the influence of adrenalin, while the other set constricts. This reaction, hereafter, will be referred to as a "differential reaction" of the blood vessels of a branchial arch.

It had been stated in earlier papers (Figge, 1930, 1934) that a change in the oxygen tension of the blood supplying the gills was at least partly responsible for gill reduction. It was, therefore, especially desirable to know how these two sets of vessels would react to changes in the oxygen and carbon dioxide tension of the blood. It was found that the two sets of blood vessels also reacted in a differential manner to both oxygen and carbon dioxide.

[1] This paper is part of a thesis submitted to the faculty of the University of Maryland Graduate School in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

For the sake of clarity and to facilitate the accurate description of the details of the physiological reactions, a brief description of a diagram of a typical branchial arch of *Amblystoma tigrinum* and a discussion of the hydrodynamics will follow.

### THE BLOOD VESSELS OF A TYPICAL BRANCHIAL ARCH IN *Amblystoma tigrinum* LARVAE

The blood vessels in the branchial arches may be divided into two groups: those forming the aortic arch proper, or primary circuit (see

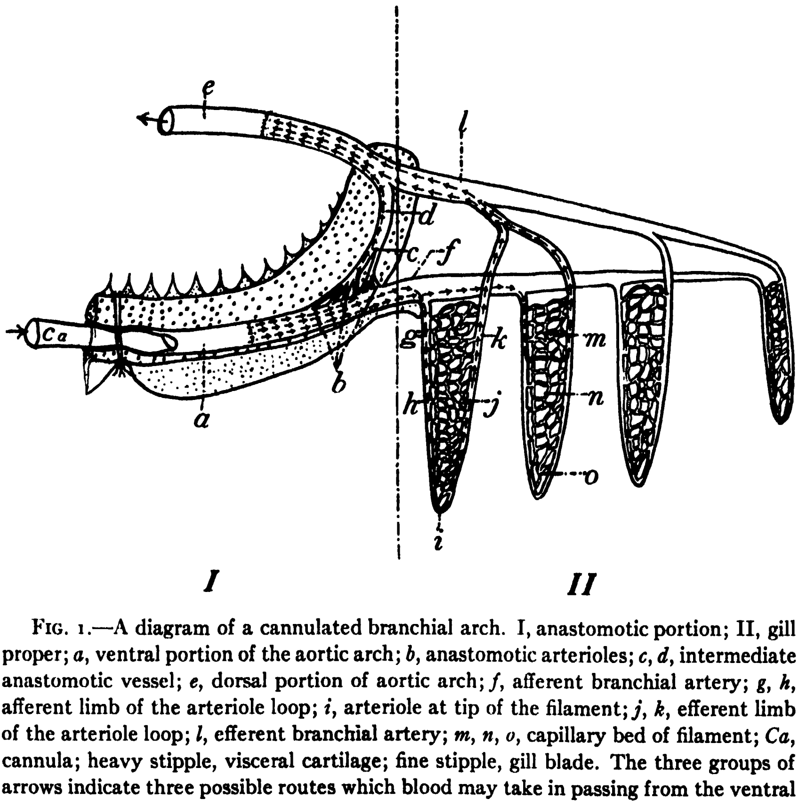


FIG. 1.—A diagram of a cannulated branchial arch. I, anastomotic portion; II, gill proper; *a*, ventral portion of the aortic arch; *b*, anastomotic arterioles; *c*, *d*, intermediate anastomotic vessel; *e*, dorsal portion of aortic arch; *f*, afferent branchial artery; *g*, *h*, afferent limb of the arteriole loop; *i*, arteriole at tip of the filament; *j*, *k*, efferent limb of the arteriole loop; *l*, efferent branchial artery; *m*, *n*, *o*, capillary bed of filament; *Ca*, cannula; heavy stipple, visceral cartilage; fine stipple, gill blade. The three groups of arrows indicate three possible routes which blood may take in passing from the ventral to the dorsal aorta.

Fig. 1); and those making up the gill proper, or secondary circuit. The aortic arch or primary circuit consists of three parts: a large ventral vessel, *a;* a large dorsal vessel, *e;* and intermediate anasto-

motic vessels, *b*, *c*, *d*. The gill, or secondary circuit, is composed of the afferent branchial artery, *f;* the arterioles, *g*, *h*, *i*, *j*, *k;* the capillaries, *m*, *n*, *o;* and the efferent branchial artery, *l*.

From the diagram (Fig. 1), it is evident that there are three ways for blood to pass from the heart to the dorsal aorta:

1. It may pass through the ventral portion of the aortic arch, *a*, and thence through the intermediate anastomosis, *b*, *c*, *d*, to the dorsal portion of the aortic arch, *e*.

2. It may pass from *a* to the afferent branchial artery, *f*, and thence through the arteriole, *g*, *h*, *i*, *j*, *k*, to the efferent branchial vessel, *l*, which communicates with *e*, the dorsal portion of the aortic arch.

3. Or it may pass through *f* to the afferent limb of the arteriole and then through any capillary in the capillary bed, *m*, *n*, *o*, to the efferent limb of the arteriole, and thence to *l* and *e*.

Under normal conditions in the larval animal some blood travels from the heart to the dorsal aorta by all three of these routes. In order for any blood to flow through the capillary bed, *m*, *n*, *o*, of the filament, the pressure must be greater in the afferent limb of the arteriole, *g*, *h*, than it is in the efferent limb, *j*, *k*.

The morphological conditions that make possible the differences in pressure in the two limbs of the arteriole are as follows: both the efferent and afferent limbs of the arteriole loop of the filament taper toward the tip, where they communicate directly with each other, so that at point *i*, the diameter of the arteriole is much less than at either *g* or *k*. It is evident that if a liquid was forced through this arteriole from *g* to *k*, the pressure would be greater at *g* and *h* than at *j* and *k*. Then, in addition to the tapering of the afferent limb of the arteriole, the viscosity of the blood and the fact that the arteriole is bent upon itself, tend to make the pressure lower in the efferent limb, *j*, *k*, than in the afferent limb, *g*, *h*.

By far the most important mechanism which controls the difference in pressure in the two limbs of the arteriole loop is the anastomosis, *b*, at the base of the gill. As long as the vessels making up the anastomosis have a small diameter, the pressure in the dorsal portion of the aortic arch, *e*, and the efferent branchial artery, *l*, may be much lower than the pressure in the ventral portion of the aortic

arch and the afferent branchial vessel. If the vessels making up the anastomosis at *b* dilate, there is a tendency to increase the pressure at *e*, *l*, and *k*, and, at the same time, to decrease it at *a*, *f*, and *g*. Dilatation of the anastomotic vessels, *b*, has a pronounced tendency to equalize the pressures in both limbs of the arteriole loop of the filament, and thus to stop the flow of blood through the filaments.

There are, then, three factors that regulate the flow of blood through the capillaries in the gill filament. One is the tone or diameter of the capillaries themselves (*m*, *n*, *o*); another is the tone or diameter of the arteriole at the tip of the filament, *i;* and last, but not least, there is the tone or diameter of the arterioles in the anastomosis at the base of the gill.

## METHODS AND MATERIALS

The animals used were normal *Amblystoma tigrinum* larvae (Colorado axolotls), one to three years old (body length, 70–140 mm.). All animals were kept in individual glass bowls of 2-liter capacity. They were fed beef liver twice weekly; occasionally earthworms were subsituted for the liver.

### METHODS AND MATERIALS FOR PERFUSION EXPERIMENTS

The animals were anesthetized in 0.1 per cent *chloretone* solution previous to the perfusion of all gills or the perfusion of an isolated gill. Unless otherwise stated, the perfusion was carried out at constant pressure of 250 mm. of water. This is slightly higher than the normal blood pressure. In order to maintain a constant pressure and determine the rate of inflow, the following method was devised: The cannula was connected, by means of rubber tubing and a T-tube for a bubble-catcher, to the bottom of a test tube. The upper end of the test tube was stoppered with a one-hole stopper, into which a 6-inch length of 8-mm. glass tubing was placed. This entire system was filled with perfusion fluid up to a mark near the middle of the 8-mm. glass tube. A burette was arranged so that the tip drained into the upper end of the glass tube. The burette was constantly adjusted, so that as fast as the perfusion fluid flowed out through the gill preparation it was replaced by fluid from the burette. The burette readings were taken at short intervals, and the amount of fluid necessary

to maintain a constant level in the tube was calculated from these. The results were then plotted from these calculations.

The gill preparations were immersed in tap water in a wax-bottom dish. The perfusion fluid was allowed to flow through the gill preparation out into the tap water through the open ends of severed blood vessels. A continuous stream of tap water was passed through the dish. This was done to maintain a constant temperature 20°–22° C. and to prevent the accumulation of adrenalin in the external medium. The preparations were tested for leaks at the end of the experiments by placing a dye in the perfusion fluid. The slightest leak could be detected by observing the outflow. A leak was found in only a few cases, and these results were discarded. The capacity of the cannula at the pressure used was measured in each case to make certain that this was much greater than the capacity of the gill that was being perfused.

In preliminary experiments all six gills of one animal were perfused simultaneously by cannulating the truncus arteriosus. The perfusion fluid was allowed to flow out of the cut ends of the dorsal aorta and the carotid arteries.

In another preliminary experiment, a single gill arch was perfused (see Fig. 1). This was removed from the animal by ligating the aortic arch ventrally as near the heart as possible, and dorsally as near the radix aortae as possible. The ventral portion of the aortic arch, *a*, was then cannulated. The perfusion fluid thus passed through either the gill or the anastomosis to reach the outflow at the cut end of the dorsal portion of the arch *e*.

To perfuse an isolated gill (without the anastomosis), the external gill was cut off just distal to the anastomosis (see point *f*, Fig. 1). The afferent branchial artery, *f*, was then split for a distance of about 2 mm. to facilitate cannulation. As soon as this vessel was cannulated, it was tied in place by a double ligature. The perfusion fluid was allowed to slowly escape from the cannula during the cannulation. The whole process of cannulation was carried out in a wax-bottom dish filled with perfusion fluid. As soon as the ligatures were tied, the full perfusion pressure was turned on. The perfusion fluid escaped through the cut end of the efferent branchial artery, *l*, In order to reach the outflow vessel, the perfusion fluid had to pass

through the gill filaments. Two isolated gills were perfused simultaneously, one with perfusion fluid alone, the other with perfusion fluid plus adrenalin. After a certain time, the two tubes were changed from one cannula to the other, so that the one that had been perfused with perfusion fluid alone was, after the exchange of tubes, perfused with perfusion fluid plus adrenalin, and vice versa.

The technique used in perfusing an isolated anastomosis was as follows (see Fig. 1): The whole gill arch was removed, as described above. The gill was severed from the visceral arch just distal to the anastomosis, *b*, leaving only a few filaments on the visceral arch portion. The ventral end of the aortic arch, *a*, was then cannulated. Any blood contained in the vessels was forced out before the afferent branchial artery, *f*, was ligated just distal to the anastomotic vessels. All the filaments on the preparation were included in the ligature. The perfusion fluid was allowed to flow out of the cut ends of the efferent branchial vessel, *e* (see Fig. 1).

The perfusion fluid employed was a modified Ringer-Locke solution that was made up fresh each day. The concentrations of the various salts were adjusted to produce a solution that was isotonic with larval salamander blood. The osmotic pressures of the perfusion fluid and of larval salamander blood were checked by the cryoscopic method. Both perfusion fluid and larval blood showed a freezing-point depression of 0.468° C. The perfusion fluid, as used, contained the following substances per liter of water: NaCl, 6.5800 gm.; KCl, 0.2650 gm.; $CaCl_2$, 0.2004 gm.; $NaHCO_3$, 0.4 gm.; dextrose, 0.9 gm.; urea, 0.5 gm.

The adrenalin chloride (1 : 1000) was obtained from Parke, Davis and Company. Adrenalin solutions were prepared just before use.

### METHODS AND MATERIALS FOR DIRECT OBSERVATION EXPERIMENTS

To confirm the results obtained by perfusion, direct observations were made on the branchial vessels of the intact animal. In order to do this, the animal was kept in shallow water and a continuous stream of water was passed through the dish. The temperature was kept at 20°–22° C. This was found to be an important factor, since the temperature, more than anything else, regulates the heart rate. The heart rate was checked before and after many experiments

to make certain that this factor was not responsible for the changes observed in the gill vessels.

The anesthesia used to immobilize the animal for making direct observations was, in most cases, nembutal, the dose depending on the size of the animal. This was injected subcutaneously in the mid-line just caudal to the gills, or, in other words, into the most cephalad part of the dorsal body fin. Nembutal was found to be far superior to chloretone, urethane, chloroform, or ether. All of the last-mentioned drugs, in concentrations sufficient to produce anesthesia, cause such great variation in heart rate and vascular tone that it was impossible to work with them. Chloretone anesthesia very frequently stops the flow of blood in the gills almost completely; this is due not to the constriction of gill vessels but to a very low heart rate. With nembutal, however, the circulation was always very good in all parts examined, and the heart rate varied only with variations in temperature. After some experimentation with the dosage, it was found possible to regulate this according to the size of the animal and the length of the anesthetic period desired. It was possible, with nembutal anesthesia, to make observations on the branchial vessels for as long a period as 10–15 hours. The animals usually recovered within 24 hours. A few curarized animals were used. In order to avoid the possible criticism that the effect observed was due to the anesthesia, a number of experiments were performed on unanesthetized animals. In the latter case, the animal was placed in a condom. This was then pinned down at various places in a wax-bottom dissecting dish. The animal was almost completely immobilized in this way and struggled very little after the first few minutes. A slit was made in the condom near the base of the animal's gills, and these were pulled out through the slit.

By placing a gill or an individual filament between a reflector (or white piece of paper) and the objective of a binocular dissecting microscope, it was possible to determine changes in the diameter of any vessel in the gill filament, even without the aid of artificial light. The changes in the diameters of the gill vessels were determined by means of an ocular micrometer. Three to five control observations were made before the application or injections of adrenalin. After the application or injection an equal number of observations were made

at various intervals. Many observations were made without anesthesia and without artificial light, but in most cases an arc light was used to facilitate the observations. The light from this was cooled and filtered by passing it through thick glass and a light-blue solution of copper sulphate. This light had no detectable effect on the vessels.

It was found possible to place one of the animal's gills in a special observation dish or tray. The small dishes or trays were trough-shaped and were made either of glass or aluminum foil. They were made just large enough to hold one gill and a small amount of the solution to be tested. The animal was pinned down in a wax-bottom dish so that the gills on one side floated near the surface of the water in the dish. One of the gills was then placed in the small tray. By this means one gill was isolated from all the other gills. The edge of the small tray over which the gill passed was fashioned out of Plasticine to conform to the shape of the gill. This prevented the fluid in the small tray from mixing with that in the large dish in which the animal and control gills were immersed. In addition, it was not necessary for the gill to bend over an edge to lie in the small tray. Any disturbance of the blood pressure or blood flow in the test gill due to bending of gill vessels over the edge of a tray was thereby avoided. Any gill could thus be immersed in solutions containing any test substance in any desired concentration. The corresponding gill on the other side was used as a control gill.

Eventually a technique was devised which permitted the application of a test substance to a single gill filament. The animal was likewise pinned down, ventral side up, in the wax-bottom dish so that the gills on one side floated near the surface. One of the gills was then supported by a wax-covered white paper. Many of the filaments were thus caused to lie flat on the wax surface. By scooping out the wax under these filaments, small pockets were made in the surface of the wax, in which the individual gill filaments rested. When small amounts of adrenalin were applied to these individual filaments, these pockets inhibited the diffusion of adrenalin to other filaments. It was thus possible to observe the effect of adrenalin solution on the blood vessels of a single gill filament. The nearby filaments were regarded as controls and observed simultaneously.

### METHOD OF REGULATING THE $O_2$ AND $CO_2$ TENSION OF THE BLOOD SENT TO THE GILLS

After the animal was anesthetized or immobilized as previously described, it was pinned down in a wax-bottom dish partly filled with salamander Locke-Ringer solution. An incision was then made in the abdominal wall just lateral to the estimated position of the tip of the lung, which was pulled out and cannulated. The lung was then pushed back into the abdominal cavity to near the normal position. The cannula was kept in position by a holder that rested on the wax in the dish. It was arranged so that either a stream of oxygen or carbon dioxide or any desired mixture of these gases might be passed through the cannula. The gas which passed through the lungs was driven out, either through the glottis or through a hole in the tip of the other lung.

The humidification of the test gases was effected by bubbling them through distilled water. Another bottle with a longer inlet tube and a higher column of water served as a pressure-regulator or safety valve, so that it was not possible for the pressure in the cannula to go above 6 cm. of water.

The effect of the variation in the oxygen and carbon dioxide tension in the blood was determined by direct observation on the gill vessels with the aid of a binocular dissecting microscope and filtered light, as described above.

## RESULTS

### A. THE EFFECT OF ADRENALIN ON THE BRANCHIAL VESSELS AS DETERMINED BY THE PERFUSION METHOD

*a*) *Perfusion of all gill arches simultaneously.*—In preliminary experiments the method employed by Keys and Bateman on eel gills, and Krawkow on fish gills, of perfusing all gills at once, was tried. It was found in these experiments that the rate of perfusion was not dependent entirely on the size of the vessels perfused but was influenced by the rhythmic contractions of the bulbus arteriosus. Moreover, the rate of perfusion did not change appreciably on addition of adrenalin. These negative results suggested that one set of vessels in the branchial circulation reacted by dilatation with adrenalin while another constricted. Because of this, the perfusion of a single gill arch and part of a gill arch was attempted.

*b*) *Perfusion of a single gill arch.*—In another preliminary experiment, a whole gill arch (external gill plus the anastomosis at the base) was perfused (see Fig. 1). A ligature was placed around the anastomotic vessel at *d* so that it could be tightened or released at will. During a control period of 40 minutes, the rate of perfusion for the entire branchial arch averaged 23 cc. per hour. When the ligature was tightened and tied securely, only the external gill or gill proper was perfused. The perfusion rate fell to 3 or 4 cc. per hour. Adrenalin was added to the perfusion fluid, and the rate of perfusion promptly increased to 15 cc. per hour. This rate was maintained for about an hour; then the gill was perfused with perfusion fluid alone. The rate of perfusion gradually fell to 6 cc. per hour. The ligature was then removed from the anastomotic vessel, and the entire branchial arch was now perfused. The rate of perfusion increased to 30 cc. per hour. Adrenalin was then added to the perfusion fluid; and this had little, if any, effect. From this preliminary experiment it was evident that, in order to test the effect of any substance on the gill vessels, it would be necessary to perfuse the gill proper and the anastomosis separately.

*c*) *Perfusion of the isolated external gill or gill proper* (without the anastomosis).—Adrenalin was used in concentrations ranging from 1:50,000 to 1:5,000,000. All were found effective and had the same action. It was tried seventy-two times on thirty-five individual gills. The gills were cannulated distal to the anastomotic vessels (see point *f*). In every case except one, the addition of adrenalin to the perfusion fluid increased the rate of perfusion. In this case, perfusion fluid a week old had been used.

The effect of the addition of adrenalin to the perfusion fluid may be described more quantitatively by the use of graphs (see Fig. 2, gills *12B* and *13B*). The results obtained from two gills that were perfused at the same time are plotted on the same paper. It will be seen that, when a gill that had been perfused with perfusion fluid alone was perfused with perfusion fluid plus adrenalin (1:1,000,000) there was a distinct increase in the rate of flow. Sometimes the increase was as great as 200 per cent. This gill was again perfused with perfusion fluid alone, and the rate of perfusion dropped to the original level.

*d*) *Perfusion of the isolated anastomosis.*—When the anastomosis at the base of a gill was isolated and perfused by the technique described, the detached gill was placed on another cannula and perfused at the same time (see Fig. 1). In order to compare the reactions, the rates of perfusion were plotted in the same graph (see

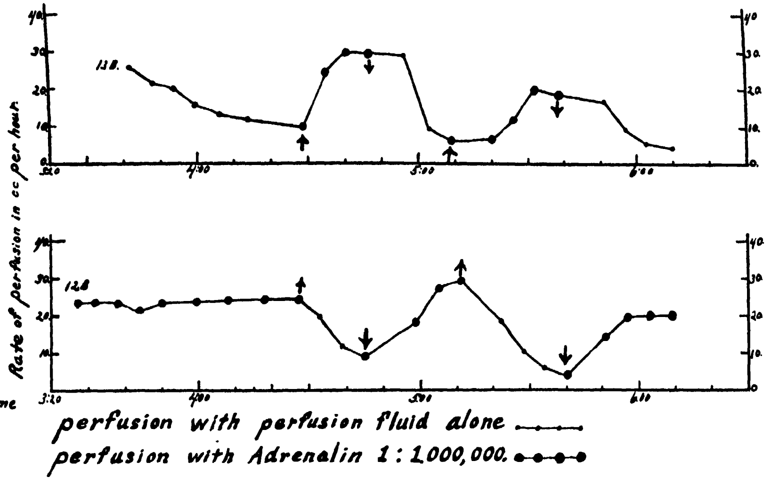


FIG. 2.—The quantitative effect of adrenalin on the perfusion rate in isolated gills (see Fig. 1). *12B*, right second gill; *13B*, right third gill. Arrows indicate the beginning and termination of periods during which the preparations were perfused with perfusion fluid plus adrenalin. These graphs show, quantitatively, that the addition of adrenalin to the perfusion fluid during the perfusion of isolated external gills causes the perfusion rate to increase.

Fig. 3). It was observed that the reaction of the anastomotic vessels to adrenalin is just opposite to that observed in the gill. When adrenalin was added to the perfusion fluid, the arterioles making up the anastomosis constricted and caused a decrease in the rate of perfusion. The effect was not as marked as the dilatation was in the case with the isolated gill but was, nevertheless, quite distinct.

The results of perfusion of a first gill and corresponding anastomosis may be seen quantitatively in Figure 3 (gill *10A* and *23B*). The curve *23B* represents the rate of perfusion in the gill proper of the first branchial arch. As stated previously for the isolated gill, perfusion with the perfusion fluid plus adrenalin (1:50,000) caused an increase in the rate of perfusion; while perfusion with perfusion

fluid alone caused a marked decrease in the rate of perfusion. The rate with adrenalin in this case was 50–60 cc. per hour. When perfused with perfusion fluid alone, the rate dropped to 34 cc. per hour.

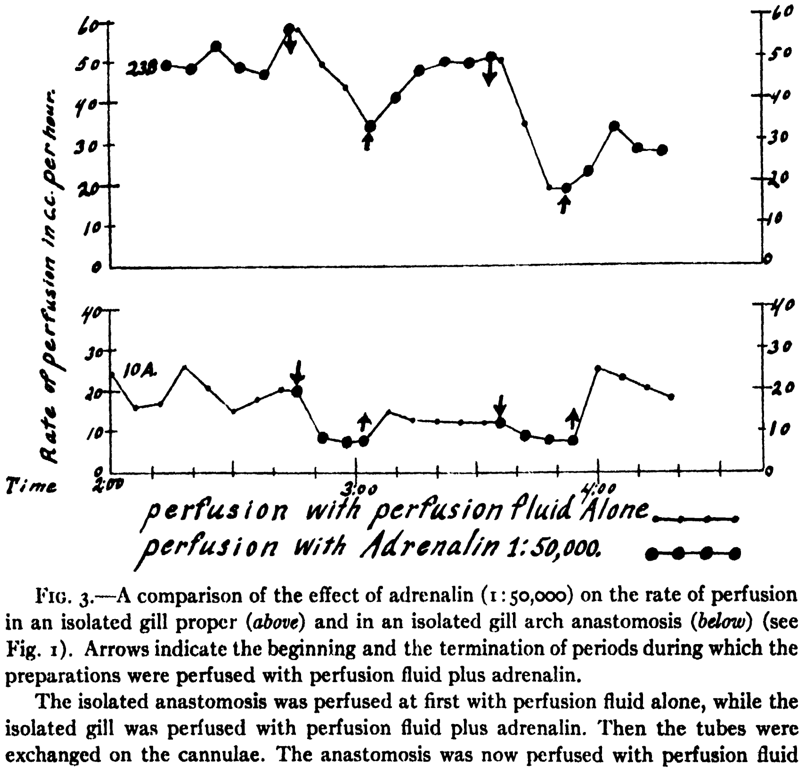


FIG. 3.—A comparison of the effect of adrenalin (1:50,000) on the rate of perfusion in an isolated gill proper (*above*) and in an isolated gill arch anastomosis (*below*) (see Fig. 1). Arrows indicate the beginning and the termination of periods during which the preparations were perfused with perfusion fluid plus adrenalin.

The isolated anastomosis was perfused at first with perfusion fluid alone, while the isolated gill was perfused with perfusion fluid plus adrenalin. Then the tubes were exchanged on the cannulae. The anastomosis was now perfused with perfusion fluid plus adrenalin, which constricted the anastomotic vessels, as evidenced by the decrease in the rate of perfusion. Meanwhile, the gill was perfused with perfusion fluid alone. This (as has already been demonstrated) caused a decrease in the perfusion rate of the gill.

The two perfusion tubes were again exchanged on the cannulae. The gill was now perfused with adrenalin, while the anastomosis was perfused with perfusion fluid alone. The rate of perfusion increased in both preparations. Then the same procedure was repeated with similar results.

It is evident from this that adrenalin constricts the anastomotic vessels at the base of the gill, while it dilates the vessels in the gill proper of the same gill arch.

Adrenalin solution was again perfused, and the rate went up to 50 cc. per hour. A change to perfusion fluid alone caused a decrease in the rate of flow to 18 cc. per hour.

During the time that the gill proper was being perfused with adrenalin, the anastomosis from the same branchial arch was being perfused with perfusion fluid alone. When the tubes were exchanged on the cannulae, the gill was then perfused with perfusion fluid alone, and the anastomosis with adrenalin solution. The reaction of the anastomotic vessels to adrenalin was just the opposite to that of the gill. The gill vessels constricted when they were perfused with perfusion fluid alone, while the anastomotic vessels constricted when they were perfused with adrenalin solution. The rate of perfusion in the anastomosis during a 50-minute period averaged about 20 cc. per hour (see Fig. 3). When perfused with adrenalin solution, the rate dropped to 7.5 cc. per hour. A change to perfusion fluid alone increased the rate to 15 cc. per hour. The rate of perfusion then gradually decreased to 12 cc. per hour. When adrenalin was again perfused, it fell to 7.5 cc. per hour. Perfusion without adrenalin solution then increased the rate to over 20 cc. per hour.

The experiment was repeated forty times on twenty-two gill anastomoses. The concentration of adrenalin ranged from 1:10,000 to 1:5,000,000. The anastomoses from all aortic arches were tested. Only five anastomoses did not respond to adrenalin by constriction. Three of these were found to have a leak in the system. One was perfused with perfusion fluid a week old. Why there was no response in the one anastomosis is not known.

### B. THE EFFECT OF ADRENALIN ON THE BRANCHIAL VESSELS AS DETERMINED BY DIRECT OBSERVATIONS

It is evident from the perfusion experiments that adrenalin constricts the arterioles in the anastomosis at the base of the gill, while it dilates the vessels in the gill itself. It was impossible to determine just which vessels within the gill proper dilated when perfused with adrenalin. To confirm the results of the perfusion experiments and, in addition, to determine which vessels in the gill reacted to adrenalin, direct observations were made on the branchial vessels of the intact animal.

The effect of adrenalin was tested thirty times on twelve different animals. The concentrations ranged from 1:2,000 to 1:1,000,000. The effect observed was always an unmistakable dilatation of the capillaries of the gill filaments. In addition to the observations that

the capillaries increase in diameter, there is other evidence in favor of a capillary dilatation.

While observing the capillaries in the tip of a gill filament, 0.2 cc. of a 1:3,000 solution of adrenalin was injected into one of the aortic arches. Strangely enough, the blood stopped flowing through the vessels at the tips of the filaments (see point *o*, Fig. 1). That this was not due to constriction of the vessels was evident. Moreover, the vessels at the tip were filled with blood which fluctuated back and forth with each heart beat. Why the blood should stop flowing through the tip of the filament was indeed perplexing. Later it was observed that the capillaries at the base of the filament had dilated to such a degree that it was possible for all the blood to pass through a few capillaries located near the base of the filament (see *m*, Fig. 1). Here it flowed quite rapidly, following the easiest and shortest route through the filament.

In addition to the microscopic observations, there is macroscopic evidence in favor of dilatation of branchial vessels. The intravenous injection of adrenalin, or the immersion of a gill in adrenalin solutions, causes the gill filaments to increase in size. The whole gill becomes distended and reddish in appearance. In one case the increase in length of a single filament was measured. Before adrenalin, it measured 2.5 mm.; afterward it measured 3.5 mm. Since each filament is composed mostly of capillaries, the increase in size may be taken as evidence in favor of capillary dilatation.

By the method of direct observation, it was possible to show conclusively that adrenalin, in all concentrations tested, produced an extreme dilatation of the gill capillaries.

### C. THE REACTION OF THE BRANCHIAL VESSELS TO $O_2$ AND $CO_2$ AS DETERMINED BY DIRECT OBSERVATION

The effect of oxygenated blood on the branchial vessels was tested thirty-five times on ten animals. The result was, in all cases, a decrease in the amount of blood passing through the gill capillaries. If the oxygen treatment was continued long enough, a complete cessation of the flow of blood in the gill capillaries resulted. This was not due to a decreased heart rate. The heart rate was taken before and during the administration of oxygen; and it was found that usually it was the same during the treatment with oxygen, occasionally less,

and sometimes even higher. Moreover, it was noted that, even though no blood flowed through the gill capillaries, it was flowing very rapidly through the capillaries in the gill blade, operculum, and the lung. The cessation of the flow of blood in the gill capillaries cannot, therefore, be due to a decrease in the heart rate or general blood pressure.

The direct observation of these vessels shows that the arteriole loop of the filament dilates in both limbs and especially at the tip (see Fig. 1). One is also able to observe an active constriction of the capillaries. Both of these reactions are difficult to describe quantitatively; but in addition to the observations on the change in diameter of the capillaries, there are other observations that may be taken as evidence of active constriction. If the capillaries were merely passive and had no way of increasing or decreasing their tone, the only factor that would cause a cessation of the flow of blood in them would be an equalization of pressures at both ends of the capillary, a condition that might be the result of a dilatation of the anastomotic arterioles, *b*. That this is not the only factor operating to stop the flow of blood in the capillaries may be seen from these facts (see Fig. 1):

1. The pressures on both ends of the capillaries are never equal as long as blood flows through the arteriole, *g*, *h*, *i*, *j*, to *k*. For, if the pressure at *g* were exactly equal to that at *k*, no blood would flow from one point to the other. The capillaries were frequently closed off while blood flowed through the arteriole loop from *g* to *k*.

2. If it were merely a matter of equalization of pressures at both ends of a capillary, we would expect all of them in one filament to close off at once. But this did not happen. In most cases, the capillaries at the base *m*, or middle *n*, of the filament closed off first, and later the capillaries at the tip of the filament. Sometimes, however, it was just the opposite. At any rate, blood did not stop flowing in all of them at once. When the lungs were ventilated with carbon dioxide and blood again began to flow through the capillaries in the filament, the last capillaries to close down were the first to open up. Different capillaries thus show differences in threshold.

We may conclude from this that there is an active constriction of the capillaries in the filament when the filament is supplied with oxygenated blood.

A portion of a protocol of one of these experiments follows:

Animal XXVI B 6, Colorado axolotl, curarized, January 13, 1934. Condition: Larval, normal. Respiratory reflexes (as evidenced by occasional gill movements) intact during the entire experiment.

| | |
|---|---|
| 3:40–4:20 P.M. | Operation: An incision made in right side, tip of lung pulled out and cannulated. Left lung was likewise pulled out and cannulated to allow the gas to escape from the tip of that lung. |
| 4:25 P.M. | Pulse rate: 57 beats in 60 sec. |
| 4:28 P.M. | Pure oxygen started through lungs. Bubbles coming out of animal's mouth and incision in left lung. |
| 4:33 P.M. | Many of the capillaries in gill filaments closing off. Heart beating as fast as ever. Respiratory movements cease. Filaments seem to be decreasing in size. Heart rate: 45 beats in 47 sec. |
| 4:35 P.M. | Gill capillaries, *m*, *n*, *o*, practically shut off, even at the base of the filaments (see Fig. 1). |
| 4:40 P.M. | Stopped oxygen and began ventilating animal's lungs with expired air. |
| 4:45 P.M. | Blood begins to flow through capillaries at tip of filament (evidence of capillary constriction at base of filament). Respiratory movements returning. Blood in fifth aortic arch becoming darker. |
| 4:50 P.M. | Gill circulation opening up. Filaments seem to be larger and more fluffy. Pulse rate: 41 beats in 43 sec. |
| 5:35 P.M. | Started oxygen through lungs, which are distended in a few seconds. Circulation in gill capillaries normal. Distension of lung with oxygen seems to dilate lung capillaries. |
| 5:37 P.M. | Blood in aortic arches has become bright light red. |
| 5:44 P.M. | Pulse rate 62 beats per 60 sec. Filaments becoming pale. |
| 5:45 P.M. | Blood still flowing through capillaries at tip of filament. Other capillaries in the filament have closed. |
| 5:49 P.M. | Even the blood in the sixth aortic arch is light red. |
| 6:00 P.M. | Capillaries in gill completely closed off. Blood flows through the arteriole loop. |
| 6:10 P.M. | Same as foregoing. Heart rate: 62 beats per 60 sec. |
| 6:20 P.M. | Condition the same. |

In general, it was observed that the capillaries in the first gill closed first, then those in the second, and that the last ones to react were those in the third gill. This may be related to the action of the spiral valve which sends most of the blood from the lungs to the first gill, a small portion of it to the second gill, and very little to the last gill and sixth aortic arch.

As it had been established that a change from venous (reduced) to oxygenated blood caused a cessation of blood flow in the gill capillaries, and that this was not due to a slow pulse, the following question arose: Was the cessation of the flow of blood through a gill due entirely to the constriction of the gill capillaries, or could this be related in any way to the reactions of the anastomotic vessels at the base of the gill?

This question was answered by making direct observations on the anastomosis at the base of the gill during oxygen treatment. Before the blood was thus oxygenated in the lungs, no blood flowed through some of the anastomotic vessels *b* (see Fig. 1); they were, therefore, not noticed until 3 minutes after the oxygen treatment was started. These, as well as all other anastomotic arterioles, dilated gradually and finally carried a wide stream of blood. Eventually, most of the blood passed directly to the dorsal aorta through the anastomosis, and very little passed through the gill. Ten per cent carbon dioxide was then passed through the lungs. The blood in the aortic arches became darker, and the arterioles in the anastomosis gradually constricted. Blood soon stopped flowing in many of the arterioles of the anastomosis. Eventually, most of the blood passed through the gill proper, and only a relatively small quantity passed through the anastomosis.

The question also arose as to whether the reaction was due to a reflex related perhaps to distension of the lungs or stimulation of afferent fibers in the gills. That the reaction was not a reflex was evident from the speed of the reaction and the long latent period. The blood did not stop flowing in the branchial capillaries suddenly. On the contrary, only a few capillaries at a time closed off, and the whole reaction took place so gradually that it sometimes extended over a period of 20–30 minutes. Meanwhile, the blood in the aortic arches, judging from the color, became more and more saturated with oxygen. The reaction time was also related to the degree of ventilation of the lungs and to the condition of the circulation in the lungs. In the second place, the reaction cannot be a reflex caused by the distension of the lungs, because, if expired air, nitrogen, and nitrogen plus carbon dioxide were used to distend the lungs, just the

opposite effect was produced. Blood circulated through the gill capillaries at a tremendous rate.

In spite of all this evidence against the possibility of the reaction being due to a reflex, the following experiment was performed. An animal was prepared and cannulated in the usual manner. All the gill capillaries were closed off by passing oxygen through the lungs. Fifteen per cent carbon dioxide was then passed through the lungs to cause blood to circulate in the gill capillaries. Having thus established the fact that the animal reacted in a normal manner, all the nerves to the lungs and gills were cut on one side. This included sectioning the vagus and glossopharyngeal nerves close to the chondrocranium. The oxygen experiment was then repeated, and observations made on the gill vessels on both the operated and unoperated side. The reaction occurred on both sides at the same rate and degree.

In another animal the same procedure was followed, and the same results obtained. Then the other lung and gill were also denervated, and the lungs inflated with oxygen. The gill capillaries again closed and reopened when the lungs were ventilated with carbon dioxide. This is additional evidence that the reaction cannot be dependent upon a reflex. It was concluded from these experiments that changes in oxygen and carbon dioxide tension have a direct effect on the blood vessels in the gills.

## DISCUSSION

The perfusion experiments show that adrenalin causes a great increase in the rate of flow through the gill proper peripheral to the anastomosis, while it causes a decrease in the rate of flow through the anastomosis. The decrease in the rate of flow through the anastomosis when perfused with adrenalin is not nearly as great as the increase in perfusion rate which adrenalin produces in the gill. It is not the magnitude of the reaction that is remarkable, but the fact that in the same gill arch we find some vessels which constrict, while others dilate, when perfused with adrenalin. If we should perfuse both sets of vessels at the same time, the reaction of one set would tend to negate the reaction of the other set. This explains why it was found impractical to perfuse all gills and gill arches at the same time.

It is interesting to note that while the reactions of the two sets of vessels are antagonistic, as far as the diameters of the vessels are concerned, they are synergistic with regard to the physiological effect on the entire animal. Any increase in the adrenalin content of the blood causes more blood to pass through the gill capillaries to be oxygenated, while less blood passes directly from the heart to the systemic vessels through the anastomosis at the base of the gill. The differential reaction produced by adrenalin would thus increase the efficiency of oxygenation of blood by the gills.

At the same time, the dilatation of the gill capillaries reduces the resistance to flow in the gill proper and would thus have a tendency to increase the blood pressure in the more peripheral systemic vessels. In this connection the results of Wyman and Lutz (1932) are interesting. These investigators found that the intravenous injection of adrenalin produced long-sustained pressor effects in *Squalus acanthias*. They measured the pressures in both the ventral and dorsal aortae. They found that the percentage increase of diastolic pressure in the dorsal aorta was consistently greater than that in the ventral aorta. They interpreted this as being due to a vasoconstrictor action of adrenalin peripheral to the gill capillaries, but admit that the region of action was not located. In my opinion, the fact that the percentage increase in the diastolic pressure in the dorsal aorta was consistently greater than that in the ventral aorta cannot be explained by a simple vasoconstrictor action of adrenalin peripheral to the gills. There must be in addition to this action a dilatation of the vessels in the gills. That adrenalin dilates the branchial vessels was shown by Krawkow (1913) in fishes and by Keys and Bateman (1932) in eels.

Probably the most interesting and fundamental fact that emerges from the present work is the observation that in the same organ, one set of vessels dilates while another constricts, when treated with the same substance. That histamine constricts the arterioles and dilates the capillaries was pointed out by Dale and Richards in 1918. The results of Dale and Richards are discussed because these investigators demonstrated a differential reaction of blood vessels to histamine and adrenalin in very low concentrations. Their results have been confirmed over and over again, but it must not be for-

gotten that the conclusion was the result not of direct observations on the capillaries but of physiological analysis. In the work presented in this paper, the differential vascular reaction to adrenalin was demonstrated by direct observation.

An increase in the carbon dioxide content of the blood going to the branchial arches also causes the anastomotic vessels to constrict and the gill capillaries to dilate. However, it might be added that, like adrenalin, it causes more blood to flow through the gill capillaries to be oxygenated, and less blood to flow directly from the heart to the dorsal aorta through the anastomotic vessels. This is, again, the result that one would expect.

The vessels of a branchial arch also react in a differential manner to changes in oxygen tension, but it should be emphasized that the reactions are just the reverse of those produced by adrenalin or carbon dioxide. Oxygenated blood causes the anastomotic arterioles to dilate and the gill capillaries to constrict.

This differential reaction of the branchial vessels, i.e., a dilatation of the anastomotic arterioles and a constriction of the branchial capillaries when they are supplied with oxygenated blood, is a truly remarkable mechanism. It prevents the waste of energy that would be necessary to force oxygenated blood through the gill. Instead of having to pass through the gill capillaries, the oxygenated blood is shunted directly to the systemic vessels through the anastomosis, *b* (see Fig. 1). It is this same mechanism that makes it possible to reduce the gills when, during a normal metamorphosis, more and more oxygen begins to enter the blood at other respiratory surfaces. This supports the hypothesis that the oxygenation of blood at points other than the gills is one of the factors responsible for gill reduction.

This recalls the attempts of Weisman (1875), of von Chauvin (1876, 1885), and of Boulenger (1913) to produce metamorphosis by enforced air breathing or by purely external non-glandular factors. Many of these attempts were doubtless successful, but later investigators have obtained varying and conflicting results (Powers, 1903; Huxley and Hogben, 1922; Huxley, 1925). One is given the impression, however, that there is something in the external environment (oxygen perhaps) that plays some rôle in the production of metamorphic changes. Recently (Figge, 1934), it was shown that it was

impossible to produce gill reduction in animals in which the sixth aortic arch was ligated, and which were kept in an environment of low air pressure (60 cm.).

The present paper explains how oxygen may influence gill reduction. Oxygenation of the blood in the lungs, skin, or pharynx as shown in this paper would cause the blood to stop flowing through the gill. In experiments that have not been published as yet, except in thesis form, it was shown that stopping the flow of blood through a gill by surgical means causes the gill filaments to be reduced. By stopping the flow of blood through a gill, the oxygenation of blood at points other than the gills is one of the factors responsible for gill reduction.

SUMMARY AND CONCLUSIONS

1. A technique was developed which permitted the perfusion of a single gill arch of *Amblystoma tigrinum.* This technique was later used to perfuse the separate parts of a gill arch. The gill proper was separated from the anastomosis at the base of the gill, and each part was perfused separately.

2. The addition of adrenalin to the perfusion fluid caused an increase of from 100 per cent to 200 per cent in the perfusion rate in isolated gills of *Amblystoma tigrinum* larvae (for anatomical diagram see Fig. 1; for result see graph, Fig. 2).

3. The addition of adrenalin to the perfusion fluid caused a decrease in the perfusion rate of the anastomosis at the base of the gill (for anatomical diagram see Fig. 1; for result see graph, Fig. 3).

4. By applying adrenalin to individual gills and parts of gills, and making direct observations on the branchial vessels of the intact animal, it was established that the capillaries in the gill filaments (*m, n, o* in anatomical diagram, Fig. 1) dilate tremendously under the influence of adrenalin.

5. This double reaction, i.e., constriction of the anastomotic arterioles at the base of the gill and dilatation of the gill capillaries of the same gill arch, which is caused by the same stimulus or substance, is called the "differential reaction" of the vessels of a branchial arch.

6. Ventilation of the lungs of *Amblystoma tigrinum* larvae with 10–15 per cent carbon dioxide induces differential reactions of the

vessels of a branchial arch which are the same as those produced by adrenalin. The anastomotic arterioles constrict, while the gill capillaries dilate. More blood therefore flows through the gill capillaries to be oxygenated.

7. Ventilation of the lungs with oxygen also induces differential reactions of the vessels of a branchial arch; but the reactions are just the reverse of those produced by carbon dioxide, i.e., oxygenation of the blood in the lungs causes the anastomosis at the base of the gill to dilate and the gill capillaries to constrict. The reactions of the two sets of vessels are antagonistic as far as the diameters are concerned but synergistic in regard to the physiological effect produced. As a result of these reactions, the oxygenated blood cannot flow through the constricted gill capillaries; it flows, instead, directly to the dorsal aorta and systemic vessels by way of the dilated anastomotic arterioles.

8. This work is presented as additional evidence in favor of the hypothesis that a change in the oxygen content of the blood sent to the gills is a factor in gill reduction.

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# REGIONAL DIFFERENCES IN EYE-FORMING CAPACITY OF THE EARLY CHICK BLASTODERM AS STUDIED IN CHORIO-ALLANTOIC GRAFTS

(Four figures and one plate)

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THE present investigation is concerned with relative eye-forming capacities of various regions of the chick blastoderm in stages from late primitive streak through head process up to and including early somite stages. It is proposed (1) to determine the position, extent, and boundaries of the area with potencies for eye production in late streak and head-process stages, (2) to analyze the relative eye-forming capacities of the various regions within this area, and (3) to consider the evidence for a changing organization with respect to eye-forming potentialities at different stages in development.

There is present in the blastoderm of chicks of late streak or of head-process stages a definite region with eye-forming capacities. It is possible to determine the level in the embryonic axis and to define with a fair degree of accuracy the boundaries of the eye-forming area under the conditions of chorio-allantoic grafting.

In normal development the optic vesicles arise by lateral outpouchings of the walls of the forebrain. If we trace development backward to late streak and head-process stages, it is apparent that the prospective eye-forming areas are bilateral in position. In amphibians, Woerdemann (1929), Petersen (1924), and Manchot (1929) have shown by using vital staining technique that the eyes arise from bilaterally situated areas.

Two interpretations have been offered regarding the position and characteristics of the eye-forming areas. Spemann (1904, 1912) advanced the view from a study of *Triton*, *Bombinator*, and *Rana* that eye-forming areas are bilaterally situated and determined at the neurula stage, and that this determination extends even to specific

parts of the eye. Ekman (1914) and Fischel (1921) support Spemann's interpretation.

According to Stockard (1909, 1913), the eye area is first median and then by cell movements of eye-forming materials becomes bilateral in position. This view is based on experimental production of cyclopia in *Fundulus* and defect experiments on the neural plate of *Ambystoma*. Adelmann (1930) has shown for the urodeles that in the medullary plate stage median and lateral regions of the anterior end of the medullary plate have the capacity for eye production. These results favor the view of a single area with eye-forming potencies which includes lateral, as well as median, regions. The greatest capacity for eye production was demonstrated to lie in the median region.

The results of the present investigation demonstrate that in chick blastoderms of late streak and early head-process stages, prospective eye-forming potencies are present in median and lateral regions at the levels of the anterior part of the node and of the anterior end of the notochord, respectively. As in the urodeles, the capacity is greater in median than in lateral pieces.

A distinction must be made here between the prospective potencies and prospective significance of certain regions for eye production, since the areas showing eye-forming capacity may not coincide exactly with the areas which actually give rise to the eyes normally. The extent to which these coincide cannot be determined from the results of the present investigation. The experiments reported here deal with the potencies and are not directly concerned with the prospective value of the various areas.

Results obtained from experiments on late streak and head-process stages, namely, that median as well as lateral regions have eye-forming capacities, led to experiments in which an attempt was made to determine at what stage in development the median region loses this capacity. The results here are of interest in that up to the nine-somite stage narrow median strips from the brain floor still gave eye tissues under the conditions of the experiment.

This work represents a portion of a larger program undertaken by Dr. B. H. Willier and his students to map out the various organ germ areas of early chick blastoderms and to study their potentiali-

ties under the conditions of chorio-allantoic grafting. The advice and criticisms of Dr. Willier, who has supervised this investigation, have been responsible in large measure for the progress made. I should like to express my appreciation of his assistance. Also, I with to express my appreciation of the excellent facilities provided at the University of Chicago and the University of Rochester which enabled me to carry out this investigation.

## MATERIAL AND METHODS

The method employed is essentially that described by Willier (1924). A chick blastoderm of desired stage in development is removed from the yolk and placed in a Syracuse watch glass. The embryo is kept in 0.9 per cent sodium chloride solution at a temperature of 39° C. throughout the operation. In a watch glass the blastoderm is flattened out, and by means of an ocular micrometer measurement of the length and width of the pellucid area and the lengths of the primitive streak and head process are recorded. These measurements, together with structural characteristics, are utilized in ascertaining the stage of the donor embryo.

Fine glass needles are employed in making the desired isolations. By placing the needle on the blastodisk and applying slight pressure, clean, accurate cuts are made with a minimum destruction of tissue. Each implant thus isolated is transferred in a drop of saline solution by means of a Spemann micropipette to the chorio-allantoic membrane of a host embryo previously incubated for 9 days. The grafted tissue becomes incorporated in the vascularized membrane of the host. After a period of 9 days, during which the graft grows and differentiates, it is recovered for histological examination. The grafts were fixed in Bouin's solution, sectioned at 8 $\mu$, and stained with Heidenhain's iron haemotoxylin.

In connection with the study of the grafts preparations of 9-day normal embryos and a complete series of whole mounts of primitive streak, head-process, and early somite stages were examined. Some exceptionally fine slides of normal embryos, both serial sections and whole mounts, were loaned to me by Dr. B. H. Willier and Miss Mary E. Rawles, of the Zoölogy Department, University of Rochester.

## DESCRIPTION OF DONORS AND ISOLATION OF IMPLANTS

The donors consisted of late streak, head-process, and early somite stages. The pellucid areas of late primitive streak stage used in the present experiments average 1.69 mm. in width, with a range from 1.26 to 2.18 mm., and average 2.67 mm. in length, with a range from 2.01 to 3.12 mm. The primitive streaks vary in length from 1.34 to 1.89 mm., with an average of 1.66 mm. The streaks in all instances are sufficiently far advanced to display definite primitive pits. The foregoing measurements are based on a total of 270 cases.

In the head-process stage the pellucid area varies in length from 2.52 to 3.57 mm., with an average of 3.02 mm.; in width the variation is from 1.47 to 2.31 mm., with an average of 1.80 mm. There is considerable variation in lengths of streak and head process, as this series includes all stages from the beginning process up to the first indication of a head fold. The range in length of head process is from 0.08 to 1.6 mm., with an average of 0.57 mm. The primitive streak ranges in length from 1.26 to 2.18 mm., with an average of 1.64 mm. Since the head process is gradually increasing and the streak correspondingly decreasing in length as a result of the backward movement of the node, the relative lengths of the two furnish a check on the developmental stage. The preceding measurements are based on a total of 372 cases.

The early somite stages used as donors comprise a continuous series of stages from presomite to twelve somites. This is the developmental period in which the morphogenesis of the neural tube is in progress. In the earliest stages (presomite to five somites) the neural folds are rolling up to form a tube. From the fifth to about the eighth somite stage a fusion of the neural folds occurs, and the walls of the diencephalon region are evaginating to form the optic vesicles. Foregut and heart are also in the process of formation during this period (Lillie, 1908, chap. v).

In late streak stages the primitive pit furnished a convenient center for describing the location of the various regions to be used as implants. The cuts are made at varying distances anterior, posterior, and lateral to the pit. A representative composite picture of the cuts made is shown in Figure 1. The distance of the cut from the pit is given in millimeters.

For purposes of analysis the letters "A," "Cb," "Ca," and "B" are assigned to the different levels of the blastoderm used in this study. It will be seen that there is considerable overlapping between the different levels. However, level A always refers to the region separated anterior to a transverse cut made in front of the pit. Posterior to this cut in each case is level Cb, which includes the pit level plus a level posterior to it. The anterior limit of the B level is at a variable distance posterior to the primitive pit. The region anterior to this level includes the pit level plus level A, and is consequently designated as level "Ca." The distance of the most posterior cut from the pit varies. An attempt was made, however, to make the areas of the levels involved in the isolation approximately equal.

Longitudinal cuts made at varying distances from the mid-line intersected the transverse cuts at right angles dividing the levels into corresponding left, median, and right pieces. The isolations are completed by making cuts around the anterior margin of the pellucid area.

Six pieces are thus isolated from each donor, as follows: left, median, and right from levels A and Cb or left, median, and right from levels Ca and B. In some instances, only the median and lateral pieces of one level are utilized.

Head-process stages are treated essentially the same as the streak stages, except that the anterior end of the notochord serves as the center about which the cuts are made. The designation of levels here is the same as in the case of streak stages (A, Ca, Cb, and B). Transverse and longitudinal cuts divide the area to be tested into left, median, and right pieces at these levels. The most posterior cut is made anterior to Hensen's node, except in short head-process stages, where it is necessary to include the node in the posterior level to maintain approximately equal areas in the levels (Fig. 2).

A third series of experiments, suggested by the results obtained from streak and head-process stages, was to isolate a very narrow strip from the median region of still older embryos to test its capacity for eye production. A piece 0.084 mm. average width (range from 0.06 to 0.1 mm.) by 0.4–0.5 mm. long was taken from the prospective brain-floor region of donors ranging from presomite to the twelve-somite stage. The notochord is always included in the piece taken. In

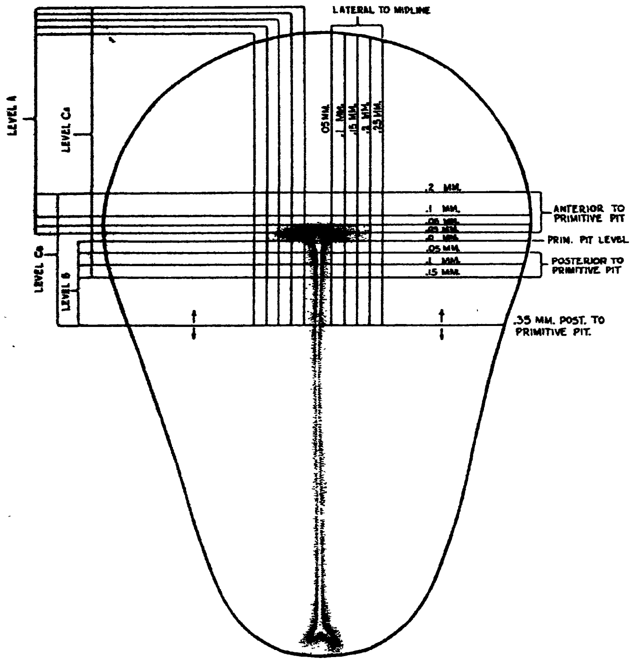


FIG. 1.—Diagrammatic representation of various cuts made in the isolation of implants at late streak stages. The position and extent of the eye area is shown. The density of stippling indicates roughly the frequency with which eye parts differentiate within the area.

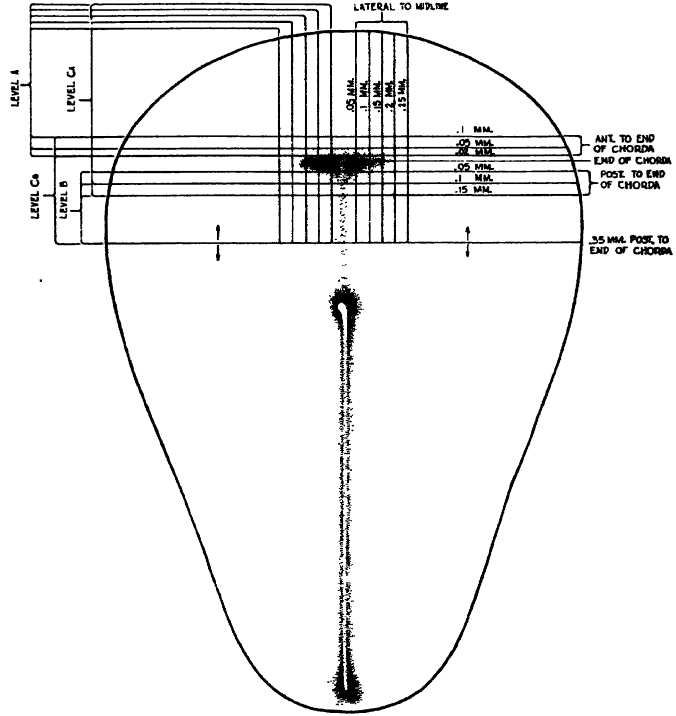


FIG. 2.—Diagrammatic representation of various cuts made in the isolation of implants at head-process stages. The position and extent of the eye area is shown. The density of stippling indicates the eye-forming frequency of various parts of the area.

some cases the head fold and developing foregut is included with the implant. After the five-somite stage the fusing neural folds must be spread apart before making the isolation.

## EXPERIMENTS CONCERNING EYE-FORMING CAPACITY

### I. POSITION, EXTENT, AND BOUNDARY OF THE EYE AREA

*At late primitive streak stages in development.*—From an examination of grafts developed from implants of various regions of late streak stages, it is determined that an area extending from the primitive pit laterally, anteriorly, and possibly posteriorly has the capacity for eye production. Figure 1 shows the eye-forming area of a late streak stage as determined by an examination of a total of 107 grafts yielding 35 cases in which eye parts differentiate.

From a total of 15 grafts obtained from implants more than 0.16 mm. lateral to the primitive pit, one shows differentiation of eye. This case resulted from a left implant of the primitive pit level isolated by a cut 0.18 mm. from the mid-line. Eight of the 13 grafts in this group were obtained from pieces 0.2–0.24 mm. from the pit. None of these 8 grafts contained eye material. It seems probable from these results that about 0.18 mm. represents the approximate lateral extent of the eye area, although the number of grafts included in the analysis is too small to establish this point.

The anterior and posterior limits are much more sharply defined than the lateral, which probably means that the level in the host axis, from which eye can differentiate, is quite definitely fixed even at this early stage in development. In 12 grafts from regions more than 0.06 mm. anterior to the primitive pit no cases of eye were recorded. Of a total of 24 grafts from 0.03 to 0.06 mm. anterior, 8, or 33 per cent, produced eye tissue. From the primitive pit to the anterior edge of the node is about 0.06 mm. Thus all these anterior regions which gave eye included a small amount of the node material.

Posterior to the pit the frequency of eye production is extremely low. Only one case has been obtained. This came from a left piece taken posterior to a cut made 0.02 mm. behind the pit level. Hunt (1932) reports no cases of eye in 25 grafts from implants taken posterior to a transverse cut through the pit.

Summarizing it may be observed that at the late primitive streak stage, any part of an ellipsoidal area extending from the primitive pit approximately 0.06 mm. anterior, 0.18 mm. lateral, and possibly 0.02 mm. posterior has the capacity to produce eye parts.

*At head-process stages in development.*—Figure 2 is a diagrammatic representation of the eye area as it relates to other regions of the chick blastoderm at the head-process stage in development. The position and extent of the area figured was determined by the examination of a total of 131 grafts, of which 56 contained eye tissue.

The area which has eye-forming capacities is located at the level of the anterior end of the notochord. Although this region has been demonstrated to have such a capacity by Hunt (1931), Rudnick (1932), and Stein (1933), its exact position, extent, and boundaries have not been determined previously.

An analysis of the data shows that the center of this area lies at or near the anterior end of the notochord and extends out from this center in all directions. To ascertain the extent of this area, cuts are varied in an antero-posterior and also in a mediolateral direction as described above. Measurements are made of the distance of each cut from the anterior end of the notochord. This variation is from 0.02 to 0.1 mm. anterior, from 0.04 to 0.2 mm. posterior, and from 0.05 to 0.24 mm. lateral to the anterior end of the notochord.

In the posterior direction within 0.1 mm. from the anterior end of the notochord 5 cases of eye were obtained from a total of 20 grafts, for a percentage of 25. Of these 5 cases, 1 was from left, 3 from median, and 1 from right regions. Beyond 0.1 mm. no eye tissue was present in 11 grafts.

The anterior limit is apparently closer than this to the anterior end of the notochord. From 13 grafts within 0.05 mm. anterior to the notochord 7 cases of eye were obtained. Here also left, median, and right regions are represented. Beyond 0.05 mm., however, no eye tissue was obtained from 5 grafts. Incidentally, it should be noted that graft frequency is low at this extreme anterior level.[1]

In a mediolateral direction the extent of the area is not so well defined; but of a total of 11 grafts from lateral pieces, 0.16 mm. or

[1] **I am indebted to Dr. B. H. Willier and Miss Mary E. Rawles for 6 grafts included in the tabulations of level A.**

more from the mid-line, only 1 case of eye was obtained. This came from a left piece beyond 0.19 mm. lateral to the mid-line. Six of the grafts were from pieces more than 0.2 mm. from the middle. This case of eye was obtained from the C level (level of the anterior end of the notochord), which may mean that the lateral extent of the area is greatest at this level.

Summarizing, it is observed that eye is obtained from regions as far as 0.05 mm. anterior and 0.1 mm. posterior to the anterior end of the notochord and as far lateral as 0.19 mm. from the mid-line. From these data a map of the area with eye-forming capacity has been made (Fig. 2). The area is somewhat ellipsoidal in outline; its transverse diameter is about 0.38 mm., and its longitudinal diameter is 0.15 mm.

The number of grafts examined is too small to permit the statement that this is the absolute limit of the area with the capacity to produce eye. Such a statement could be based only on negative results in large numbers of grafts from regions outside the area outlined. However, it seems reasonable to conclude that the area defined above includes by far the larger portion of the region with eye-forming potencies, as expressed in chorio-allantoic grafts.

The limitations of the method make it impossible to determine whether a narrow strip of cells in the median line lacks the capacity to produce eye. However, grafts obtained with both pigmented and sensory layers of the retina from median strips less than 0.08 mm. wide or one-twentieth the width of the pellucid area makes it seem highly improbable that a strip lacking this capacity is present.

A comparison of the areas with eye-forming capacity in late streak and head-process stages shows that they are similar as to size, shape, and extent. However, the area is located at the primitive pit in the late streak stage and at the anterior end of the notochord in the head-process stage. When we consider the manner of formation of the notochord (i. e., by the backward movement of the primitive knot, leaving a strand of cells behind), it becomes apparent that the two positions are entirely comparable as to level in the axis. The medio-lateral extent of the area is practically the same for both stages. However, the antero-posterior extent is much greater in the

head process than in the late streak stage. This may be accounted for by the fact that during the formation of the head process there is an elongation of the blastodisk in an antero-posterior direction. The evidence presented above would seem to show then that the two areas are comparable as to position, extent, and boundaries.

### II. FREQUENCY DIFFERENCES IN EYE-FORMING CAPACITY WITHIN THE EYE AREA

The eye differentiates in grafts from both median and lateral regions at the level of the anterior end of the notochord at head-process stages and the level of the node in late streak stages. The median regions have a greater capacity for eye production than do the lateral pieces. This is revealed in these grafts in two ways: first by a study of the relative eye frequencies, and second by an analysis of the morphogenesis and histogenesis of the eye.

*At late primitive streak stage.*—Table I summarizes the data on the relative frequencies of the various regions within the eye-forming area. From median pieces of levels A, C, and B (Fig. 1) 48 per cent, or 19 of the 40 grafts studied, contained eye material. Left pieces from the same levels produced eye material in 27 per cent, or 10 of the 37 grafts recovered; while 6 grafts (20 per cent) of the 30 obtained from comparable right regions showed differentiation of eye tissue. From the C level alone the eye-frequency is higher in both median and laterals, i. e., median 81 per cent, left 44 per cent, and right 43 per cent. However, the relation between medians and laterals remains about the same as for all levels taken together.

From this analysis, two important differences in eye-forming capacity are observed: first, the median is higher than either of the laterals; and second, the left is higher than the right. Not only is there a difference in eye-forming frequency, but a corresponding difference in frequency of grafted piece to survive. The median region is highest (45 per cent), left next (40 per cent), and right lowest (34 per cent).

Carrying the analysis still farther, it may be seen that ability of grafts to survive, as well as frequency of eye-differentiation from lateral pieces, tends to decrease as the distance of the lateral piece from the mid-line increases (Table I).

A comparison of frequency of eye differentiation in level C (the level of the primitive pit) and levels anterior and posterior to it shows a decrease in eye-forming capacity in both directions. The decline is much more rapid per given distance, anteriorly and posteriorly from the pit, than has been observed in a medio-lateral direction. This is shown in Table I, where it may be noted that from 0.03 to 0.06 mm. anterior to the pit the frequency of eye formation is 33 per cent, as compared with 57 per cent for the pit level. Posterior to the

TABLE I

EYE-FREQUENCIES FROM MEDIAN AND LATERAL PIECES AT VARIOUS TRANSVERSE LEVELS (LATE PRIMITIVE STREAK STAGE)

| | | Level A Anterior to Primitive Pit | | | | | | Level C Primitive Pit | | | Level B Posterior to Primitive Pit | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Distance of Cut Anterior and Posterior to Primitive Pit | | .07-2 mm. | | | .03-.06 mm. | | | .00 mm. | | | .00-.05 mm. | | | .06-.16 mm. | | | Totals | | | Percent | | Average Percent | |
| | Distance of Cut from Midline | Hosts Alive | Number of Grafts | Number of Eyes | Hosts Alive | Number of Grafts | Number of Eyes | Hosts Alive | Number of Grafts | Number of Eyes | Hosts Alive | Number of Grafts | Number of Eyes | Hosts Alive | Number of Grafts | Number of Eyes | Hosts Alive | Number of Grafts | Number of Eyes | Grafts | Eyes | Grafts | Eyes |
| Left | .16-.24 mm | 3 | 2 | 0 | 2 | 1 | 0 | 13 | 5 | 1 | 2 | 0 | 0 | 9 | 1 | 0 | 29 | 9 | 1 | 31 | 11 | 40 | 27 |
| | 11-.15 mm | 6 | 2 | 0 | 11 | 4 | 2 | 13 | 8 | 3 | 7 | 4 | 0 | 4 | 2 | 0 | 41 | 20 | 5 | 49 | 25 | | |
| | 05-10 mm. | 4 | 0 | 0 | 0 | 0 | 0 | 9 | 3 | 3 | 5 | 3 | 1 | 5 | 2 | 0 | 23 | 8 | 4 | 35 | 50 | | |
| Median | .16-.24 mm | 2 | 1 | 0 | 2 | 1 | 1 | 6 | 3 | 2 | 0 | 0 | 0 | 4 | 1 | 0 | 14 | 6 | 3 | 43 | 50 | 45 | 48 |
| | .11-.15 mm. | 7 | 3 | 0 | 17 | 8 | 4 | 15 | 9 | 7 | 7 | 2 | 0 | 2 | 1 | 0 | 48 | 23 | 11 | 48 | 48 | | |
| | .05-.10 mm. | 4 | 2 | 0 | 3 | 3 | 1 | 10 | 4 | 4 | 3 | 1 | 0 | 6 | 1 | 0 | 26 | 11 | 5 | 42 | 45 | | |
| Right | .05-.10 mm | 3 | 1 | 0 | 0 | 0 | 0 | 12 | 6 | 3 | 4 | 1 | 0 | 3 | 2 | 0 | 22 | 10 | 3 | 45 | 30 | 34 | 20 |
| | .11-.16 mm. | 6 | 1 | 0 | 15 | 6 | 0 | 12 | 5 | 3 | 6 | 0 | 0 | 4 | 2 | 0 | 43 | 14 | 3 | 33 | 21 | | |
| | .16-.24 mm. | 2 | 0 | 0 | 1 | 1 | 0 | 10 | 3 | 0 | 2 | 0 | 0 | 9 | 2 | 0 | 24 | 6 | 0 | 25 | 00 | | |
| Totals | | 37 | 12 | 0 | 51 | 24 | 8 | 100 | 46 | 26 | 36 | 11 | 1 | 46 | 14 | 0 | 270 | 107 | 35 | | | | |
| Percent grafts | | 32 | | | 47 | | | 46 | | | 31 | | | 30 | | | 4 | | | | | | |
| Percent eyes | | 00 | | | 33 | | | 57 | | | 9 | | | 00 | | | 33 | | | | | | |

pit the drop is even more abrupt; i.e., to 9 per cent within 0.05 mm. of the pit. In fact, no eye material was obtained from grafts beyond 0.02 mm. posterior to it.

*At head-process stage.*—As described above, cuts made on each side of the mid-line at distances varying from 0.05 to 0.24 mm. isolated left, median, and right regions. By varying the cuts in this manner, it is possible to see what effect the inclusion or exclusion of a certain region has on the presence of certain structures and also on the differentiating capacities of the various areas. This method has been utilized here to test the difference in eye-forming capacity of various regions.

A tabulation of the case numbers from left, median, and right regions of levels A, C, and B according to the distance of the cuts from the anterior end of the notochord is contained in Table II. The frequencies are given in percentages. An analysis of these results shows that eye frequency is highest in the median (52 per cent), next in left (43 per cent), and lowest in right (27 per cent). There is a gradual increase in eye frequency of lateral pieces as the region includes more of the median area. On the left this increase is from 20 to 48

TABLE II

EYE-FREQUENCIES FROM MEDIAN AND LATERAL PIECES AT VARIOUS TRANSVERSE LEVELS (HEAD PROCESS STAGE)

| | | LEVEL A ANTERIOR TO NOTOCHORD | | | | | | LEVEL C ANTERIOR END OF NOTOCHORD | | | LEVEL B POSTERIOR TO ANTERIOR END OF NOTOCHORD | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| DISTANCE OF CUT FROM ANTERIOR END OF NOTOCHORD | | .06-1 MM | | | .02-.05 MM | | | .00 MM | | | .04-.1 MM | | | .1-.2 MM. | | | TOTALS | | | PERCENT | | AVERAGE PERCENT | |
| | DISTANCE OF CUT FROM MIDLINE | HOSTS ALIVE | NUMBER OF GRAFTS | NUMBER OF EYES | HOSTS ALIVE | NUMBER OF GRAFTS | NUMBER OF EYES | HOSTS ALIVE | NUMBER OF GRAFTS | NUMBER OF EYES | HOSTS ALIVE | NUMBER OF GRAFTS | NUMBER OF EYES | HOSTS ALIVE | NUMBER OF GRAFTS | NUMBER OF EYES | HOSTS ALIVE | NUMBER OF GRAFTS | NUMBER OF EYES | GRAFTS | EYES | GRAFTS | EYES |
| LEFT | 16-.24 MM. | 5 | 1 | 0 | 7 | 0 | 0 | 15 | 3 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 29 | 5 | 1 | 17 | 20 | | |
| | .11-.15 MM. | 11 | 1 | 0 | 5 | 1 | 0 | 38 | 11 | 7 | 1 | 1 | 0 | 5 | 2 | 0 | 60 | 16 | 7 | 27 | 44 | 33 | 43 |
| | .05-.10 MM | 0 | 0 | 0 | 3 | 1 | 1 | 26 | 15 | 9 | 8 | 4 | 1 | 7 | 3 | 0 | 44 | 23 | 11 | 52 | 48 | | |
| MEDIAN | .16-.24 MM | 4 | 1 | 0 | 5 | 2 | 2 | 17 | 10 | 7 | 2 | 1 | 0 | 0 | 0 | 0 | 28 | 14 | 9 | 50 | 64 | | |
| | .11-.15 MM. | 9 | 1 | 0 | 6 | 1 | 1 | 32 | 14 | 10 | 2 | 1 | 0 | 5 | 1 | 0 | 54 | 18 | 11 | 33 | 61 | 42 | 52 |
| | .05-.10 MM. | 0 | 0 | 0 | 3 | 2 | 1 | 23 | 11 | 4 | 12 | 6 | 3 | 8 | 3 | 0 | 46 | 22 | 8 | 48 | 36 | | |
| RIGHT | .05-.10 MM. | 0 | 0 | 0 | 5 | 3 | 2 | 20 | 5 | 3 | 6 | 3 | 0 | 6 | 1 | 0 | 37 | 12 | 5 | 32 | 42 | | |
| | .11-.15 MM | 9 | 1 | 0 | 6 | 1 | 0 | 29 | 10 | 3 | 2 | 2 | 1 | 3 | 1 | 0 | 49 | 15 | 4 | 31 | 27 | 30 | 27 |
| | .16-.24 MM. | 6 | 0 | 0 | 4 | 2 | 0 | 14 | 3 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 25 | 6 | 0 | 24 | 00 | | |
| TOTALS | | 44 | 5 | 0 | 44 | 13 | 7 | 214 | 82 | 44 | 35 | 20 | 5 | 35 | 11 | 0 | 372 | 131 | 56 | | | | |
| PERCENT GRAFTS | | 11 | | | 30 | | | 38 | | | 57 | | | 31 | | | 36 | | | | | | |
| PERCENT EYES | | 00 | | | 54 | | | 54 | | | 25 | | | 00 | | | 43 | | | | | | |

per cent, and on the right from 0.0 to 42 per cent. As the size of the lateral piece increases, the corresponding median decreases. This results in a corresponding decrease in frequency in the median pieces. The difference in frequency may be due to the small size of the narrowest median piece grafted.

Parallel with the differences in eye frequencies are differences in graft frequencies, particularly between median and laterals; i.e., median pieces produce grafts in 42 per cent of cases, left in 33, and right in 30. That this is not merely a chance occurrence is emphasized by corresponding differences observed in similar transplants

of late streak stages. Apparently, medio-lateral differences exist in the capacity to produce graft, as well as in capacity to differentiate eye parts.

An analysis of the differences in eye-forming capacity of different levels emphasizes the importance of level in determining potentialities for organ formation. A comparison of eye-forming frequency of grafts from the level of the anterior and of the notochord (C level) and levels anterior (A level) and posterior (B level) to it shows that there is a marked decrease in both directions from the level of the anterior end of the notochord. Eighty-two grafts from level C gave eye material in 44 cases (54 per cent), as compared with 18 grafts yielding 7 cases of eye from level A and 20 grafts from level B, 5 of which contain eye. These figures include grafts from left, median, and right pieces isolated by cuts less than 0.1 mm. either anterior or posterior to the anterior end of the notochord.

These results, when combined with those described above for medio-lateral differences, show a grading-off in all directions from a region of relatively high eye-forming capacity to regions which entirely lack this capacity (Table II). The narrow antero-posterior extent of the eye area, plus the fact that the limits in both directions are quite well defined, indicates that the level for eye production has been established before this stage in development.

Figure 3 is a diagrammatic representation of these differences in terms of the distance from the mid-line of the cuts which separated the respective left, median, and right regions. The data used in constructing these graphs are summarized in Tables I and II. Although the curves do not show a smooth grading-off from the mid-line laterally, the tendency in each case is clearly indicated. The drop in frequency of formation of eye tissue from right regions is much more abrupt than from left regions, although a definite decrease is evident in both directions. The percentage values for corresponding regions in the two stages do not agree in all instances; but a definite similarity in the general trend of the two curves is evident; i.e., highest in the median, intermediate in height on the left, and lowest on the right.

Curves of the relative frequencies of the different levels from streak, as well as head-process, stages are shown in Figure 4. The

sharp decrease in each case is probably indicative of the importance of level in the host axis in determining the structures which differentiate. The difference in the sharpness of the decline in the two

FIG. 3

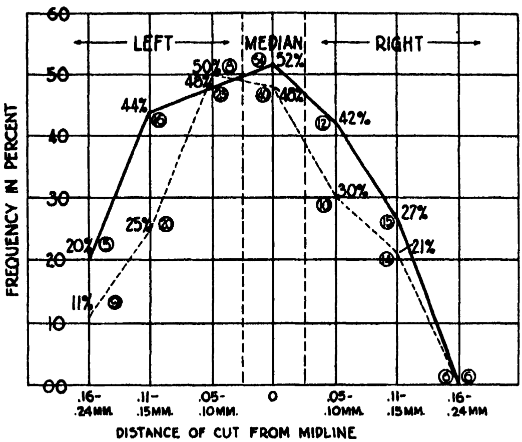


FIG. 4

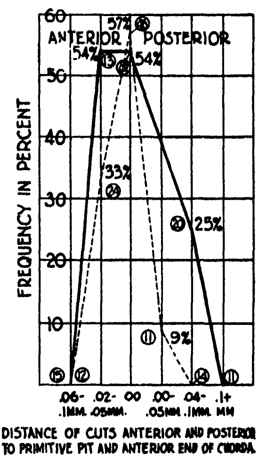


FIG. 3.—Graphic representation of the medio-lateral gradient in eye-forming frequency at late streak and head-process stages.

FIG. 4.—Graph showing the relative eye-forming frequencies of levels anterior and posterior to the primitive pit in late streak stages and the anterior end of chorda in head-process stages. (Late streak stages are represented by broken lines, and head-process stages by unbroken lines. The encircled figure is the number of grafts examined.)

cases may be accounted for partially at least by the fact that the blastoderm elongates considerably in the head-process stage, whereas the width is not increased proportionately.

### III. EYE-FORMING CAPACITY IN EARLY SOMITE STAGES

The percentage of eye frequency for median regions at late streak stages (Table I) is 48, as compared with 52 at head-process stages (Table II), an increase of 4 per cent; while for left regions an increase of 17 per cent (from 27 to 43) and for right regions an increase of 7 per cent (from 20 to 27) is observed. A graphic representation of this relative difference between medians and laterals at these two developmental periods is contained in Figure 3. This figure is evidence of a gradual increase in eye-forming capacity in lateral regions. Both median and lateral regions show greater eye-forming capacity

in the later stages, but the rate of change is more rapid in the laterals than in the medians.

This line of evidence led to an analysis of the eye-forming capacity in younger and older stages than those just analyzed. The question as to how eye-forming potentialities of median and laterals compare in stages younger than those described above remains for future investigation. An attempt has been made, however, in the present investigation to analyze the condition in embryos still older than those considered above. For this purpose donors varying in age from presomite[2] to twelve somites were used. A piece of the brain floor was taken out at the level of the forebrain, and usually extending posterior to it. Pieces grafted were from 0.06 to 0.10 mm. in width by 0.4–0.5 mm. in length. They include in each case the underlying notochord. In some cases the head fold and foregut material was removed, and in others it was transplanted with the brain-floor material. What influence, if any, the removal of foregut has on the differentiation of eye is not revealed by the present experiments. Eye differentiated in grafts from pieces without foregut material.

TABLE III

EYE-FREQUENCIES FROM BRAIN FLOOR GRAFTS

| STAGE | HOSTS ALIVE | No OF GRAFTS | No OF EYES | PERCENT GRAFTS | PERCENT EYES |
|---|---|---|---|---|---|
| 1-3 SOMITE | 17 | 6 | 4 | 35 | 67 |
| 4-8 SOMITE | 17 | 7 | 5 | 41 | 71 |
| 9-12 SOMITE | 9 | 4 | 0 | 44 | 00 |
| TOTALS & AVERAGES | 43 | 17 | 9 | 40 | 53 |

IMPLANTS RANGED IN WIDTHS FROM .063-.10MM

It is of interest to note that all of the grafts resulting from this extremely small implant are very small, but the differentiation is not noticeably different histologically from that in much larger grafts.

For convenience in making comparisons the grafts are divided into three groups according to the stage of the donor from which they came. Table III summarizes concisely the results obtained from each of these groups. The data show in the first place that eye is produced with a relatively high frequency in stages from presomite to eight somites. The percentages are approximately the same for the presomite to three-somite and the four-to-eight-somite groups. Secondly, it will be observed that from nine-to-twelve-somite stages

[2] "Presomite" is used here to refer to the stage in which the neural folds are beginning to arise but before the first somite is definitely indicated.

no cases of eye were obtained from 4 grafts. This result indicates that the eye-forming capacity, which is present in the brain-floor region from presomite to eight-somite stages, is lost during the following stages. A great many more grafts would be necessary to establish this point, but the results are of sufficient interest to justify their inclusion in this paper. It has been demonstrated, at least, that the capacity for eye production remains in this median region, even up to the stage at which the optic vesicles are beginning to form. A definite isolation of the potencies into the lateral primordia does not precede the beginning of the morphogenesis of the optic vesicles.

It is interesting to note here that in a series of 6 grafts of the lateral region, which is prospective eye material, eye tissue was present in every graft. When this result is compared with that for head-process stages, it becomes evident that a definite rise in the eye-forming potentiality of the lateral regions has occurred during this developmental period.

### IV. QUALITATIVE DIFFERENCES WITHIN THE EYE AREA AS SHOWN BY A STUDY OF MORPHOGENESIS AND HISTOGENESIS

Regional differences in capacity to produce eye is very evident in the grafts studied in the present investigation. It is not my purpose in the present paper to consider in detail the qualitative differences in the various regions, but to describe more or less typical cases and to summarize the differences in capacity for differentiation of the regions under consideration.

Eye tissues differentiating in grafts do not display normal relationships, probably because of mechanical factors operating during morphogenesis. The histology, however, is essentially like that of normal tissues. This observation is substantiated by comparing the results of Weysse and Burgess (1906) on the normal histogenesis of the retina and of Hoadley (1925) on the histology of eye tissues in grafts.

Analysis of regional differences in eye-differentiating capacity in grafts from both head-process and late streak stages shows that the median region has a much greater capacity to form essential eye tissues consisting of sensory and pigmented layers of the retina and lens than do the lateral regions. However, in grafts from median regions all degrees of differentiation are present from a small amount of

## PLATE I

FIG. 5.—A portion of an optic cup showing the various layers of the differentiated retina. The implant was a median piece of donor at head-process stage (see text for details). ×235.

FIG. 6.—An optic cup showing both pigmented and sensory layers of the retina; differentiated from a median piece of donor at late streak stage (see text for details). ×210.

FIG. 7.—A large optic vesicle showing lens in contact with convoluted pigmented layer; differentiated from a left anterior piece of donor at head-process stage (see text for details). ×38.

FIG. 8.—A portion of a graft showing convoluted pigmented layer connected with forebrain material; differentiated from a right anterior piece of donor at head-process stage (see text for details). ×140.

PLATE I

pigmented layer to a definite optic cup with the differentiated retina. In Figure 5, a section of such an eye cup is shown. The donor from which the grafted piece was taken was in the head-process stage. The implant included the anterior 0.1 mm. of the notochord, 0.1 mm. on each side of the mid-line and extending anteriorly to the margin of the pellucid area.

Out of a total of 28 cases of eye tissue recorded from median pieces, 6 showed a differentiation comparable to the above mentioned case. In several other cases a much thickened layer, presumably retina, was found connected with the pigmented layer.

Figure 6 is a photomicrograph of a relatively normal-appearing optic cup. The donor in this case was at a late streak stage. The piece used as the implant was taken 0.06 mm. anterior to the primitive pit and included 0.10 mm. on one side and 0.12 mm. on the other side of the mid-line, as determined by the position of the pit. The piece extended anteriorly as far as the margin of the pellucid area.

Differentiation has not progressed as far as in the preceding case described, but the sensory layer is cupped back into a pigmented layer. In no instance was differentiated sensory layer obtained from late streak stages. This is probably a chance occurrence, as complete differentiation has been obtained from stages much younger than these (Butler, 1935).

The best cases of lens differentiation have been obtained from median regions. Three such cases have been positively identified. It is interesting to note that the ectoderm included with the implants in these cases does not normally contribute to the lens. In each of the three cases the lens is connected to skin in the graft. One possible explanation of the occurrence of lens in these grafts would be induction by the eye differentiated in the graft. In this connection it is also interesting to note that the eye tissue is derived from cells which normally do not contribute to eye formation.

When we proceed now to a consideration of the type of differentiation obtained from lateral pieces, it may be observed that no case of differentiated sensory layer has been recorded from any lateral region. However, definite vesicles are common from left lateral regions. Figure 7 shows a large vesicle entirely surrounded by pigmented layer, with a small lens shown at one point in contact with

the pigmented layer. This lens is continuous with definite skin in another section. Also in another section the pigmented layer is continuous with a cupped sensory tissue which is connected with the forebrain material seen in the center of the vesicle.

Figure 8 is quite typical of the differentiation found in right regions. Pigmented layer is present in a much convoluted form with no definite organization into an eye. It seems significant that in no case was a vesicle of the type quite common to grafts from left and median regions found.

It is also interesting to note that the quantity of eye material is smaller from right pieces than from other regions tested, and that in no case has lens been identified in grafts from right pieces but that three cases have been noted from left. The eye material is shown connected to a forebrain vesicle in the figure. This connection is typical in grafts obtained from left and median, as well as right, regions. The presence of eye in connection with nervous tissue is used as a criterion for identifying forebrain (Willier and Rawles, 1931).

By the foregoing analysis of the differentiation of eye tissue in grafts from median, left, and right regions, additional evidence is furnished for some fundamental difference in these three regions. The median region produced the highest frequency of both eye tissue and grafts and the best differentiation of eye in the graft. Furthermore, the percentage of graft survival, as well as eye formation and the type of differentiation obtained, was higher from left than from corresponding right regions. The fact that all three lines of evidence point in the same direction indicates that we are dealing with a general difference in activity of the three regions. This may be expressed as a medio-lateral gradient with a more rapid decline to the right than to the left.

## DISCUSSION

The analysis of results has brought forth three generalizations with respect to eye-forming capacities of early chick blastoderms. First, in late streak and head-process stages an area is present more or less definitely localized and limited, any part of which has a capacity to produce eye tissue. Second, within this area there exists a medio-lateral, as well as an asymmetrical, difference in eye-forming capacity. Third, during the normal developmental processes there is

a progressive increase in the eye-forming capacities of lateral regions (the regions which normally produce eye). The median region (prospective brain-floor material) does not lose this capacity until after the eight-somite stage.

### I. THE NATURE OF THE AREA WITH EYE-FORMING CAPACITY

It has been determined that the entire blastoderm at head-process stages in development is composed of organ-forming germ areas Rawles and Willier, 1934; (Willier and Rawles, 1935; and Rawles, 1935). The potential eye area then is only one of a great many such areas which overlap each other.

It has been shown previously that eye-forming capacity is present in different regions of the blastoderm at late streak stages (Hunt, 1932; Stein, 1933) and at head-process stages (Hunt, 1931; Rudnick, 1932; Stein, 1933). The present investigation has attempted to localize more definitely and to limit these regions.

The eye-forming germ area occupies a somewhat comparable position in both late streak and head-process stages. At late streak stages the primitive pit and at head-process stages the anterior end of the notochord are centers about which materials have eye-forming capacity. In both, the region with eye-forming capacity is ellipsoidal in outline with the greater diameter at right angles to the longitudinal axis of the embryo. The longitudinal diameter at late streak stages is much shorter than it is at head-process stages. Cells within these ellipsoidal areas have eye-forming capacities. In addition, these same cells may have the potentiality for various other ectodermal structures characteristic of the given level or region in which they are located. The determination process up to this time, then, is probably concerned only with restricting the area with potentialities for eye production, and not specifically and irrevocably determining certain cells for a specific organ. Potential forebrain material occupies approximately this same region and is found associated with eye in grafts from implants of any part of this area. Apparently, any part of the region, then, has the capacity to produce more than it does produce normally.

Since both sensory and pigmented layers of the retina and lens can be produced from various parts of this region, it is likely that the area is an equipotential system for eye production; i. e., any part is

capable of producing a complete organ, though very much reduced in size. Spemann and Bautzmann (1927) and Holtfreter (1931) point out that the presumptive medullary plate is equipotential. Adelmann (1929*a*, 1929*b*, 1930) has shown that in the urodeles the anterior end of the medullary plate is an equipotential system. In his experiments median or lateral regions of the anterior medullary plate were removed, and the remaining parts developed eyes. Also, these same areas transplanted separately to the belly wall produced eyes. By studying the eye-forming frequency of median and lateral pieces, Adelmann found that median regions have a much greater capacity for eye production than do laterals.

On the basis of what has been said, we cannot consider the eye area in chick embryos of these stages as either localized distinctly median (Stockard, 1913) or as bilateral primordia specifically determined (Spemann, 1912), but as an area occupying both median and lateral regions of the prospective neural plate material at the level of the primitive pit or the anterior end of the head process. The prospective eye regions in the chick are probably bilateral, as Woerdemann (1929), Petersen (1924), and Manchot (1929) have demonstrated for amphibians; but this certainly does not refer to potential singleness or doubleness of the eye anlage.

## II. MEDIO-LATERAL AND ASYMMETRICAL DIFFERENCES IN EYE-FORMING CAPACITY

It has been pointed out that the median regions produce both grafts and eyes with greater frequency than do the laterals. Also, the type of differentiation of eye in grafts from median regions is better than from the laterals, as based on the quantity of eye material, the degree to which differentiation has progressed, and the relative normality of the eyes. It has been noted that a corresponding difference is present between left and right regions; the left is superior to the right in graft and eye production, as well as in type of differentiation.

The fact that the variation is in the same direction; i. e., best in median, next in left, and poorest in right, with respect to all three of these conditions in both late streak and head-process stages furnished additional evidence that some one generalized condition is responsible for all three results.

Graft frequency can be related to a difference in the capacity of the implants to become incorporated in the chorio-allantoic membrane or to differentiate once they become incorporated. Assuming a more or less constant medium of differentiation, the difference in the capacity of the various regions to produce grafts is a function of the activity of the cells implanted. Similarly, the capacity a graft has to produce all organs for which it has potentialities or to produce a particular organ every time depends on the general ability of the cells to express all their potentialities. This would determine the frequency with which any organ would occur in grafts.

Hyman (1927) has shown by susceptibility experiments that the node region in late streak and the corresponding region of the anterior end of the head process in head-process stages are the regions of greatest metabolic activity. The results of the present experiments bear this out, using the capacity for eye production as a measure of this difference. In addition, the present experiments indicate strongly that the left side is more active than the right; but on each side there is a gradual decrease in activity in a medio-lateral direction. Using eye frequency as a measure, there is a medio-lateral gradient in cell activity, with the decline more rapid to the right than to the left.

On the basis of the discussion just presented, Figure 3, showing graphically the relative eye frequencies of left, median, and right regions, may be offered as an indication at least of the relative differences in activity of the three regions.

## III. EVIDENCE OF A CHANGING ORGANIZATION

In the preceding discussion an analysis has been made of the differences which exist in different parts of the eye area at head-process and late streak stages in development. This leads next to a consideration of the differences which exist between successive stages in the developmental process.

The data presented in the preceding sections give evidence of a regular and orderly change in the eye-forming capacities as development proceeds. An increase in the eye-forming capacity of lateral regions at head-process stages over lateral regions at late streak stages is indicated by comparison of eye frequencies. Also, the in-

crease observed in median regions from late streak to the head-process stage is not nearly as great as the lateral increase (cf. Tables I and II). When head-process stages were compared with still later stages; i. e., early somite stages, it was found that the capacity of the lateral regions (potential optic vesicles) had increased to 100 per cent, whereas the corresponding medians (potential brain floor) did not increase to more than 70 per cent. In stages beyond eight somites no eye tissue was present in 4 grafts from the brain floor.

These results are interpreted as indicating a shift in developmental potency for eye production from the median to the lateral regions. This process probably continues until the median region no longer has any eye-forming capacity, while the lateral regions produce eye every time the transplanted tissue develops into a graft. This is probably the stage in which the prospective eye areas attain specific potencies (Lillie, 1929). From this stage on, eye tissue alone would be produced by these areas, according to this interpretation.

During a comparable stage in urodeles prechordal mesoderm is shifting laterally in position, and mandibular arch material is being added from the neural crest (Adelmann, 1934). Cyclopia accompanies the failure of the prechordal mesoderm to shift laterally. Probably the activity of the mesoderm material brings about an increase in the activity of the lateral regions to an optimum for eye production, and consequently the greatest eye-forming capacity becomes located in the bilateral optic vesicles.

This explanation is supported by certain experiments in producing eye abnormalities in *Planaria dorotocephala* (Child, 1916, 1920, 1921, and 1924, pp. 107–10). By subjecting the animals to inhibiting agents during regeneration, it was found that the medio-lateral gradient could be inhibited to varying degrees so that the median region with the highest metabolic rates would be reduced and the bilateral organs approximated to the median line. In other words, the median region now assumes the metabolic rate characteristic of the bilateral organs (in this case eye), so that the eyes differentiate at or near the mid-line. If this interpretation is correct, there is a shift of eye-forming potentialities toward the mid-line as a result of an inhibition of the medio-lateral gradient; whereas in normal development the potentially single eye-forming area becomes divided into

bilateral primordia as a result of a stimulating influence exerted on the ectoderm, probably by increased mesodermal activity.

Wright and Wagner (1934), in accounting for head abnormalities in guinea pigs, picture the existence of various centers of activity at certain critical stages in development. The optic field, which is single and median, is one of these centers. If inhibitions, either genetic or environmental, are supplied to this center at the critical period, the physiological processes by which this field produces bilateral eyes is interrupted, resulting in varying degrees of cyclopia, depending on the strength of the inhibitor and the exact time of its operation.

This analysis of the cause of cyclopia is interesting in the light of the present investigation, which has shown that the median region in early developmental stages of chick blastoderms has the greatest capacity for eye production. If certain physiological processes necessary for the production of bilateral eyes were inhibited, the result would be a morphological expression of the physiological condition in existence at the time of the inhibition; i. e., the production of a single median eye. Should an inhibitor be applied at a stage in which the eye-forming capacity is greatest in the lateral regions (during the process of the formation of the optic vesicles in the case of the chick), the result possibly would be a reduction in the size of the bilateral eyes, microphthalmia, and not cyclopia. Wright and Wagner (1934) have pointed out that an inhibitory process after the separation of the two optic primordia is probably the cause of microphthalmia.

## SUMMARY

1. Grafts from implants from various regions of the pellucid area of embryos at late streak and head-process stages in development have been examined for eye production. In late primitive streak stages, an eye-forming area has been found which extends 0.06 mm. anterior, 0.02 mm. posterior to the primitive pit, and 0.2 mm. on each side of it. A corresponding area is located for head-process stages, extending 0.05 mm. anterior, and 0.1 mm. posterior to the anterior end of the head process, and 0.2 mm. on each side of it.

2. Both median and lateral regions within this area have the

capacity to produce the essential components of the eye cup, retina and pigmented layers, and a lens.

3. As a result of a comparative study of eye-forming frequency and type of differentiation in grafts from median and lateral regions, it seems apparent that there is a medio-lateral gradient in capacity for eye production, a greater capacity residing in left than in right regions.

4. During the developmental process a change occurs in the relative developmental capacities of median and lateral regions for eye production. As a consequence, at early somite stages the capacity of the lateral regions (primordia of optic vesicles) is greater than that of the median (primordium of the brain floor). The median region of the brain floor retains its potentiality for eye production up to the eight-somite stage, beyond which this potency is apparently lost.

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# Physiological Zoölogy

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## THE PHYSIOLOGY OF THE FORMATION OF "PIGEON'S MILK"[1]

(Two plates)

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IT HAS long been known that males, as well as females, of pigeons and doves produce in their crop a milky substance, commonly known as "pigeon's milk," which is fed the young for the first few days after hatching. Investigations on the subject have been, in the main, of a histological and cytological nature. Only recently have attempts been made to discover the factors inducing the crop changes and the mechanism by which they are brought about. The investigations described in this paper deal with the changes in the crop under various experimental conditions.

The pigeons used in these investigations were from the stock maintained by the Department of Genetics of the University of Wisconsin for studies on inheritance.

For histological examination of the crop Bouin's fluid was found more satisfactory than Zenker's. Not only was Bouin's easier to handle, but more precise fixation and staining were possible by its

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use. For general staining Harris' haematoxylin was most frequently used, though Delafield's also gave good results. Heidenhain's iron haematoxylin was employed for a more specific study of the nucleus. For the relation of connective tissue to other tissues Mallory's triple stain was used. Flemming's strong solution, followed by safranin, was employed to differentiate special cytoplasmic structures, especially fat droplets.

## THE NORMAL HISTOLOGICAL CHANGES IN THE CROP DURING INCUBATION AND FEEDING OF THE YOUNG

The histological changes in the crop, especially during incubation, are presented exhaustively by Litwer (1926) and Beams and Meyer (1928, 1931). A brief review is necessary, however, in order to stress some of the important changes which have been used as landmarks in judging crop growth obtained under experimental conditions.

The wall of the crop in the non-brooding bird is thin, transparent, and of yellow-white color, and consists of four coats: the outer (basal) fibrous, the muscular, the submucosa, and the mucosa (Plate I, Fig. 1). The outer, loose fibrous tissue envelops the wall and unites it to the adjacent organs. The muscular coat contains an outer longitudinal and an inner circular layer of muscle fibers. The submucosa consists of areolar connective tissue with many blood vessels and is covered by a layer of loose connective tissue, tunica propria, containing irregularly branched blood vessels, from which the mucous coat draws its nourishment. The deep layers, stratum germinativum, of the stratified epithelium adjacent to the tunica propria, consist of young, columnar, deeply staining cells, the significance of which in the hypertrophy of the crop during incubation is justly pointed out by Litwer (1926). The line of demarcation between the stratum germinativum and the tunica propria is not uniform but shows recurring smooth and zigzag areas; the latter are due to the protrusions of tunica propria into the stratum germinativum. This condition is, however, absent in the sections of the median portion of the crop, and it is suggested by Litwer that this may account for the fact that this part of the crop remains unaltered during incubation. The epithelial cells of the crop at this stage, fixed in Flemming's solution, are invariably free of lipoid droplets. This structure of the crop persists during the first 7 days of incubation.

From the eighth to the thirteenth day the walls of the lateral pouches manifest marked macroscopic and microscopic changes. Particularly by the twelfth day there is an increase in vascularity and a slight hypertrophy of the wall. Folds are noticeable even on the eighth day of incubation (Plate I, Fig. 2), and by the twelfth day their size is remarkably increased. Sections show an augmentation in the connective tissue and vascularity of the protrusions of the tunica propria into the folds of the stratum germinativum.

With the further development of the folds, accompanied by thickening of the epithelium, the spaces between them become obliterated (Plate I, Fig. 4, and Plate II, 10), while secondary ramifications of the tunica propria into the stratum germinativum, carrying capillaries with them, result in a structure superficially glandlike in appearance (cf. Plate II, Fig. 7).

The hypertrophy at this stage is accordingly brought about largely by the excessive cell multiplication in the stratum germinativum. By the eighth day lipoid droplets begin to appear in the epithelial cells and increase as the hypertrophy continues. In the meantime the median portion of the crop remains unchanged.

The incubation period of the pigeon is about 18 days. From the thirteenth day of incubation to the fourth day after hatching of the young the changes in the crop proceed with remarkable rapidity, as is evident from Plate I, Figures 3–5. The lateral pouches are rosy red in color, owing to increased vascularity, and exhibit extraordinary hyperplasia, which can be felt by palpation through the skin. The "milk" is found in the crop on about the seventeenth day of incubation. During this period the histological changes are more marked than those in the preceding period, and consist, generally, in an enormous hypertrophy of the epithelium (Plate I, Figs. 3, 4), in an increase in size and number of lipoid droplets in the hypertrophied cells, and finally in a desquamation of the surface cells of the thickened epithelium. The rate of cell multiplication in the stratum germinativum further increases, and there is an indication that the cells of the intermediate layers may divide amitotically. This is presumably associated with an early stage of degeneration due to a scarcity of nutriment, since these cells are relatively at a greater distance from the source of nutrition. The distribution of lipoid droplets in the cells of the thickened epithelium is uneven; the young columnar

## PLATE I

(Photomicrographs)

FIG. 1.—Section of the wall of a lateral pouch of the crop of a non-brooding bird. The basal layer is the fibrous (*fibr.*); next the muscular (*musc.*) showing bundles of both longitudinal and transverse fibres; the areolar submucosa (*sub. mu.*) with its covering of vascular tunica propria (*t. p.*), which supplies nourishment to the mucosa, consisting of the more deeply lying stratum germinativum (*str. ger.*) and the stratified epithelium (*ep.*), lining the lumen of the crop. Note that the epithelium is wrinkled but not definitely folded. ×50.

FIG. 2.—Section of the pouch wall on the eighth day of incubation. The epithelium is thrown into deep folds. Note the greater stratification of the epithelium and the vascularity of the protrusions of tunica propria into the epithelial folds. The increased thickness of the crop wall is at this stage due almost entirely to the depth of the folds. ×50.

FIG. 3.—Section of pouch wall at fourteenth day of incubation. The epithelium has increased greatly in thickness. ×50.

FIG. 4.—Section of the pouch wall on the third day of feeding of the young. Here there is still greater hypertrophy of the epithelium, and the process of desquamation of epithelial cells (liberation of "milk") is evident. ×20.

FIG. 5.—Enlarged view of portion of Figure 4 showing the sloughing-off of masses of epithelial cells. ×125.

FIG. 6.—Section of "milk" from the crop of a young squab. Note that the outlines of the nucleated epithelial cells are still evident. ×125.

## PLATE II

(Photomicrographs)

FIG. 7.—Section of the crop of ♂2348A on the nineteenth day of incubation. This bird was prevented from sitting on the eggs from the fifth day of brooding onward but was in sight of his mate during that time. The crop hypertrophy is normal for full incubation (cf. Figs. 3 and 4). ×20.

FIG. 8.—Section of the crop of ♂1743R on the nineteenth day of incubation. The actual time of incubation was, however, cut down by three periods of removal. This retarded the crop development, which approximates that of the tenth or eleventh day of normal uninterrupted incubation. ×50.

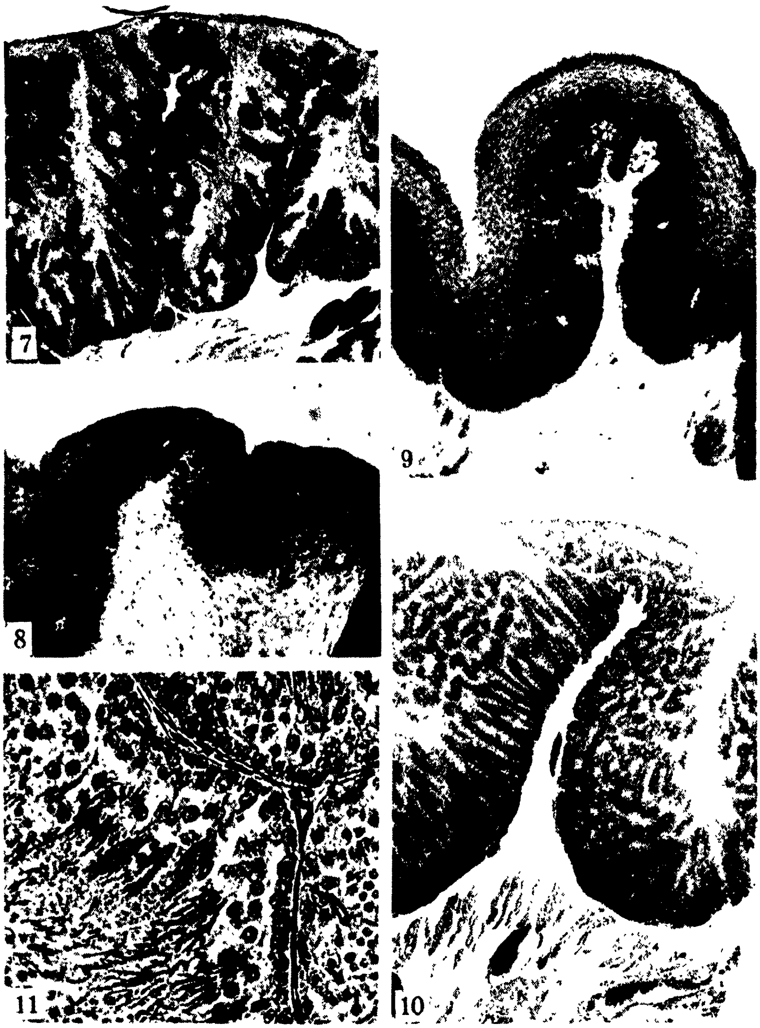
FIG. 9.—Section of the crop of the same bird (♂1743R) on the twenty-fifth day of prolonged incubation. The development has reached the stage normally attained at the fourteenth day (cf. Fig. 3). ×50.

FIG. 10.—Section of crop of same bird (♂1743R) on twenty-ninth day of incubation. Epithelium greatly thickened and desquamation commencing. Compare with the normal situation after 3 days of feeding young (Fig. 4). ×20.

FIG. 11.—Section of testis of ♂2501G on sixteenth day of incubation. Shows normal stages of spermatogenesis, with no indication of quiescence or regression. ×250.

PLATE I

PLATE II



cells of the stratum germinativum are free of these droplets, while the cells nearest to the lumen of the pouch contain more and larger lipoid droplets than those farther away. As a result of increased growth of the epithelium of the folds, the space occupied by the connective tissue and the blood vessels of the tunica propria in the fold is obliterated to a mere passage for the blood vessels (Plate I, Fig. 4).

The desquamation of the surface epithelial cells occurs over the entire area of the pouches and usually involves groups of relatively few cells. It is not uncommon, however, for several surface layers of the epithelial cells to slough off into the lumen in large masses (Plate I, Figs. 4 and 5). The histological picture of the "milk" obtained from the crop of a squab (Plate I, Fig. 6) approximates that of the epithelial cells which are ready to slough off (Plate I, Fig. 5). In both cases the cells are polygonal, with flattened nuclei, around which are vacuoles representing dissolved lipoids.

The processes of growth and desquamation in the pouches of the crop continue until about the eighth or ninth day of feeding, following which the crop returns to normal by the fourth week of feeding. Marked hyperplasia of the epithelium still exists even on the sixteenth or seventeenth day of feeding, but no "milk" is found in the crop.

With reference to the glands of Teichmann (1889), located where the crop joins the lower part of the esophagus, our observations indicate that they are functionally active even outside the feeding period. While these glands may supply enzymes (Dulzetto, 1927*a*, 1927*b*, 1928*a*, 1928*b*) or amino acids (Carr and James, 1931), their increased secretion of mucus during the feeding period probably aids also in the regurgitation of the milk.

## RÔLE OF INCUBATION IN MILK FORMATION

The facts presented in the preceding section indicate that the formation of the milk in pigeons is due to physiological factors associated with incubation. The *modus operandi* of the incubation instinct and the nature of the physiological processes underlying it are, however, little understood. An experimental procedure, therefore, to determine the rôle of incubation in the formation of the milk should consist in modifying the act of incubation and in observing the effects of such alterations on the phenomenon.

In pigeons the reproductive cycle consists of five discrete terms, which Herrick (1907) groups as cyclic instincts. These instincts follow in a definite sequence; and if the cycle is broken at any point, the whole process is abandoned and the bird begins the cycle anew. The incubation instinct is preceded by the instinct of courtship and mating, of nest-building, and of egg-laying; while the instinct of feeding and care of the young follows it.

A comprehensive description of the normal incubation behavior in pigeons is presented by Whitman (1919). The incubation period —from the time of the laying of the first egg to the hatching of the young—is 17–19 days. The male, however, does not commence incubating regularly until the appearance of the second egg. After the laying of the second egg both birds participate regularly in the act. At night the female sits on the eggs, while the male roosts away from the nest. During the day the male and female alternate in the act; the male ordinarily occupies the nest from about 10:00 A.M. to 4:00 P.M. The initiative in leaving or resuming the nest is taken by either bird. When the nest is relinquished by one bird, this acts as a signal for the other bird to resume the nest duty, and this is done immediately.

## SEPARATION OF PARENTS DURING INCUBATION

### EXPERIMENTS 1 AND 2: SEPARATION OF ONE BIRD OF PAIR TO ADJACENT CAGE BUT WITH VIEW OF MATE

In Experiment 1 the males of nesting pairs were separated from the females at different stages of incubation and placed in pens adjacent to their mates, with only a wire-netting partition between. In Experiment 2 the females were removed in a similar manner. The separated bird and the brooding mate were thus within sight of each other.

In Table I will be found a record of Experiment 1. It will be seen that, although the males were prevented from incubating eggs for 7–16 days, in all cases the normal crop changes were obtained. Plate II, Figure 7, shows the histological picture of the crop of ♂2348A at the end of the normal incubation period. The changes are similar to those obtained at the same time in birds that have incubated normally for the same period (cf. Plate I, Fig. 4). The

females as a rule incubated consistently for the remainder of the period after the male was removed. The separated male, frequently found in the corner of his cage nearest the brooding female, reacted to the movements of the female. Whenever she abandoned the nest, either to feed or to defecate, the male made evident attempts to get into the other pen, as if to occupy the nest. The resumption of the nest by the female quieted the male immediately.

A record of Experiment 2, in which females were separated, is given in Table II. These results differ from those of Experiment 1 in

TABLE I

EXPERIMENT 1

Showing the effect on the crop growth at the end of normal incubation period in males separated from, but left in sight of, their mates, after having incubated eggs for varying numbers of days

| No. of Male | Stage of Incubation When Separated (Days) | Period of Separation (Days) | Stage of Incubation When Crop Examined (Days) | Condition of Crop |
|---|---|---|---|---|
| 2192E | 12 | 7 | 19 | Normal growth |
| 2348C | 10 | 8 | 18 | Normal growth |
| 2300S | 7 | 13 | 20 | Normal growth |
| 2348A | 5 | 14 | 19 | Normal growth |
| 2281S | 5 | 14 | 19 | Normal growth |
| 2317.1 | 3 | 16 | 19 | Normal growth |
| 2283R | 3 | 16 | 19 | Normal growth |

lack of uniformity. In females 2326T, 2036A, and 2378A the changes in the crop were normal, while they were incomplete and variable in the rest of the females. In 2274R the condition was similar to that normally found at the twelfth or thirteenth day of incubation. The crops of 2326C, 2327G, 2273D, 2433B, and 2370E indicated little or no change.

After the isolation of 2326T, her mate occupied the nest for the rest of the incubation period. On the fourth day after separation the male did not attend very closely to incubation, but resumed with full intensity when the eggs hatched the next day. The behavior of this female while separated resembled that of the males in Experiment 1. In the case of 2036A and 2378A the mate continued sitting

on the eggs till the end of the normal incubation period, and the behavior of these females approximated that of 2326T. The eggs were neglected by the males in the rest of the cases before the normal incubation period terminated. The mates of 2274R, 2326C, 2327G, 2273D, 2433B, and 2370E continued brooding for 2, 2, 4, 1, 3, and 2 days, respectively, after these females were isolated. Abandonment of the nest by the male induced a change in the behavior of the female. She showed lack of interest in the activities of her mate and reverted completely to the non-brooding behavior.

TABLE II

EXPERIMENT 2

Showing the effect on the crop growth at the end of normal incubation period in females separated from, but left in sight of, their mates, after having incubated eggs for varying numbers of days

| No. of Female | Stage of Incubation When Separated (Days) | Period of Separation (Days) | Stage of Incubation When Crop Examined (Days) | Remarks |
|---|---|---|---|---|
| 2326T | 13 | 6 | 19 | Normal growth |
| 2036A | 12 | 7 | 19 | Normal growth |
| 2378A | 12 | 6 | 18 | Normal growth |
| 2274R | 13 | 6 | 19 | Incomplete growth |
| 2326C | 10 | 9 | 19 | Incomplete growth |
| 2327G | 10 | 9 | 19 | Incomplete growth |
| 2273D | 10 | 9 | 19 | Incomplete growth |
| 2433B | 9 | 9 | 18 | Incomplete growth |
| 2370E | 8 | 10 | 18 | Incomplete growth |

The results of Experiment 1 alone might be taken to indicate that once the development of the crop is initiated, actual incubation, at least for a period longer than 3 days, is not necessary for full histological development and production of milk. In the case of the females, however, the development of the crop was incomplete if the birds were removed before 12 or 13 days of incubation (Expt. 2). This difference may reasonably be attributed to the fact that the mates of the females deserted the nest within 2 or 3 days after removal of the females, whereas, when the males were removed, the females continued incubation for the full period.

EXPERIMENT 3: SEPARATION OF MALE TO ANOTHER ROOM

In this experiment the males were placed in pens in another room, where they were unable to see their mates. Table III records the results, which show variability proportional to the elapsed time of incubation when the male was separated. Even in the males 2509B, 1953E, and 2345H, isolated as late as the twelfth day of incubation, the crop development was far from complete. The epithelium was thrown into folds, but there was no increase in its thickness. This is

TABLE III

EXPERIMENT 3

Showing the effect on the crop growth at the end of the normal incubation period in males separated from the sight of the brooding females, after having incubated eggs for varying numbers of days

| No. of Male | Stage of Incubation When Separated (Days) | Period of Separation (Days) | Stage of Incubation When Crop Examined (Days) | Remarks |
|---|---|---|---|---|
| 2509B | 12 | 7 | 19 | Slight change |
| 1953E | 12 | 7 | 19 | Slight change |
| 2345H | 12 | 7 | 19 | Slight change |
| 2345F | 10 | 9 | 19 | Slight change |
| 2274P | 10 | 9 | 19 | Slight change |
| 2329H | 3 | 16 | 19 | No change |
| 2348A | 3 | 16 | 19 | No change |

comparable to the condition on the eighth or ninth day of normal brooding (Plate I, Fig. 2).

The separated males were unusually quiet for 2 days, following which their behavior reverted to the normal non-brooding type. There was no uniformity in the behavior of the females left with the eggs. Incubation was completed by the mates of 2509B, 2345H, and 2329H, while those of 1953E, 2345F, 2274P, and 2348A occupied the nest for 2, 5, 3, and 6 days, respectively, after the removal of their mates.

Kaufman (1932) reports an experiment apparently in conformity with the foregoing results.

In Experiments 1 and 2 the behavior of the separated birds of either sex resembled in some respects that of normal incubation.

Apparently, the act of the mate leaving the nest stimulated the separated bird in the same manner as when the two were together, and it may accordingly be argued that it was therefore in a similar psychological state. For convenience the behavior of the separated bird may be referred to as "psychological brooding," which persists only so long as its mate remains faithful to the nest.

### EXPERIMENT 4: SEPARATION OF MALE TO ADJACENT PEN BUT WITHOUT VIEW OF MATE

The isolated males in this experiment were kept in pens adjacent to the brooding females as in Experiment 1; but a partition of thick cardboard, which eliminated the possible influence of sight, was placed between the birds. Five males were separated after respective incubation periods of 2, 4, 4, 3, and 6 days.

No change in the crops of these males was manifest at the end of the normal incubation periods. The behavior of the separated males, after 2 days of isolation, was similar to that in Experiment 3. The behavior of the females was again variable. Two occupied the nest till the end of the normal incubation period, while the others deserted their eggs on the second, fifth, and seventh day of separation, respectively. These results confirm those of Experiment 3, that sight of the brooding mate is involved in "psychological brooding."

### EXPERIMENT 5: EFFECT OF STRANGE FEMALE ON MALE

Whether or not the influence of the sight of the brooding mate on the isolated bird can be inhibited in any way was tested by employing a strange female as a disturbing influence, for it was thought that this might lead the male to court and mate with the new female. The male was isolated as in Experiment 1, with only wire netting between; but a female, stranger to the male, was put with him. Three males were treated in this way, with varying results. The first, separated on the fourth day of incubation, paid no attention to the strange female. The crop hypertrophy was less than normal, the histological picture being similar to that of the twelfth or thirteenth day of normal incubation. The brooding female occupied the nest till the end of the incubation period. The second male was isolated on the third day of incubation. The new female introduced was aggressive, and frequently she and the male were observed fighting.

This did not, however, affect the incubating female, which continued normal incubation. The crop of the male showed no hypertrophy at the eighteenth day. The third male was separated on the fourth day of incubation and the next day was observed with the new female sitting on a nest provided for them. Two days later this female laid, but both eggs became broken; following this the male was observed frequently driving the female. At the time of completion of normal incubation by the original female the wall of the crop of the male was thin and transparent, like that of a non-brooding bird.

From the results of this experiment it is obvious that association with a new female inhibits, at least partially, the changes in the crop of the isolated male. It seems probable that the reaction of the male to the new female interrupts his state of "psychological" broodiness and hence stops the course of crop development.

EXPERIMENT 6: SEPARATION OF MALE FOR VARIOUS INTERVALS

The normal incubation period in pigeons, as has been mentioned, is about 18 days, or approximately 432 hours. The male normally devotes about one-fourth of this time, say 108 hours, to sitting on the eggs; and the female sits the remaining 324 hours. This may vary considerably under different circumstances. That the number of hours spent in actual brooding is not in itself the determining factor in crop hypertrophy is indicated by the fact that the development is complete in both sexes by the time the eggs are due to hatch. In the three cases now to be discussed the males were isolated as in Experiment 3 but were put back with their mates at intervals for short periods of varying length. As previously noted, when the males are removed early, the females tend to desert the nest. At such times it was necessary to return the males in order to have incubation continued. These periods of removal were found to have a rather definite effect on the male, depending primarily on the length of the periods during which he was isolated. Several trials were made, but only those carried to the end of the normal incubation period will be considered.

In Case A the first egg was laid on September 20 and the birds were examined on October 8, after 18 days of incubation, when the crops of both birds contained milk and showed some degree of hyper-

trophy. During the incubation period the total time that the male was removed from the nesting-pen, not counting a short interval on September 28, was approximately 207 hours in four intervals of 47, 48, 64, and 48 hours. His separation for this time did not modify the normal crop development.

The length of the period that the male was not with the female and the length of the intervals of isolation were increased in Case B. The time was cut down by 319 hours, in three periods of 111, 112, and 96 hours. This altered markedly the normal course of the changes in the crop of the male. At the end of the normal incubation period a piece of the crop was removed for histological examination. This showed the epithelium thrown into folds and slight hypertrophy (Plate II, Fig. 8). The capillaries from the vessels of the tunica propria had penetrated and entangled the cells of the stratum germinativum. These changes approximate those normally observed on the tenth or eleventh day of incubation. The reduction of incubation during the 18-day period in this case definitely retarded the normal changes. This raised the question whether crop development in the male could be continued by prolonging incubation to compensate for the reduced period. Infertile eggs were accordingly substituted before the original eggs hatched, and the birds were allowed to continue incubation as long as they would, the male now being left in all the time. By this means the incubation was extended over a period of 28 days—fully 10 days beyond the normal. After 150 hours the crops of both birds were again examined, and a piece from the male fixed for histological examination. This showed increased development, with the folds enlarged and the epithelium markedly thickened, but no milk (Plate II, Fig. 9). The histological picture resembles that of the thirteenth or fourteenth day of normal incubation (Plate I, Fig. 3). Complete crop growth and formation of the milk were, however, found when the male was killed and examined after an additional 96 hours. The epithelium was then enormously hypertrophied, and desquamation apparent (Plate II, Fig. 10). The extension of the brooding period by 246 hours thus corrected the retarding effect of the 319-hour reduction.

Case C repeated the experimental procedure of Case B. The normal incubation period for the male was shortened by 224 hours in

three intervals of 64, 104, and 56 hours. As a result of this decrease the changes in the crop of the male were not complete on the eighteenth day. There was a well-marked hypertrophy of the epithelium comparable to that obtained on the twelfth or the thirteenth day of

TABLE IV

EXPERIMENT 6

Showing effect of prolonged incubation following interruption in the case of the male

| Case | | 17–18 Days | | 20 Days | | 22 Days | | 24 Days | | 28 Days | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Brooding Time* (Hours) | Crop Development | Brooding Time (Hours) | Crop Development | Brooding Time (Hours) | Crop Development | Brooding Time (Hours) | Crop Development | Brooding Time (Hours) | Crop Development |
| A | ♂ | 225 | Normal hypertrophy *Milk* | | | | | | | | |
| | ♀ | 432 | Normal hypertrophy *Milk* | | | | | | | | |
| B | ♂ | 113 | Slight hypertrophy No milk | | | | | 263 | Normal hypertrophy No milk | 359 | Normal hypertrophy *Milk* |
| | ♀ | 432 | Normal hypertrophy *Milk* | | | | | 582 | Normal hypertrophy *Milk* | 678 | Not incubating |
| C | ♂ | 208 | Marked hypertrophy No milk | 280 | Increased hypertrophy No milk | 328 | Normal hypertrophy *Milk* | | | | |
| | ♀ | 408 | Normal hypertrophy *Milk* | 480 | Not examined | 528 | Normal hypertrophy *Milk* | | | | |

* This indicates the number of hours the bird was in the cage with nest, not actual time sitting on nest. This time was divided between the female and the male when the latter was not separated.

normal incubation. Normal development was induced, as in Case B, by extending the incubation period by an additional 120 hours.

The results obtained in Experiment 6 are summarized in Table IV. In Case A the length of the separation periods ranged from 47 to 64 hours, and yet the crop changes were complete at the end of the normal incubation period. There is no evidence to show just how long after separation regression of crop development commences, but it is evident that, in this case at any rate, such changes had not gone

far enough to retard appreciably the time of milk formation. When the intervals exceeded 96 hours, as in Cases B and C, it was necessary by way of compensation to prolong the incubation beyond 18 days in order to secure the complete crop development. The most important fact disclosed by this experiment is that even during a single incubation it is possible several times to inhibit and to initiate again the changes in the crop by controlling the act of brooding, thus delaying the normal changes.

### RELATION OF YOUNG TO THE FORMATION OF THE MILK

The fact that the formation of the milk coincides with the hatching of the young at the end of 17 or 18 days of brooding has led some investigators to speculate on the effects of the appearance of the young on the process. Thus Craig (1908) states: "In the very act of breaking the shell of the egg and emerging therefrom, the young dove begins to exert control over its parents; for the movements of the young excite the parents, confirm their attachment to the nest and its contents, and stimulate them to secrete the 'pigeon's milk' in the crop and to perform the feeding movements." At another place in the same paper this author further states: " . . . . ; after 14 days of brooding [he refers to doves], when the eggs hatch, both birds begin suddenly to secrete in the crop a food aptly called 'pigeon's milk'. . . . ." Whitman (1919, p. 66) observes: "The special secretions in the pigeon's crop seem to be stimulated in part by the sight of the young. . . . ."

A systematic study has been made to determine the effects of the young on the formation of the milk, (1) at the end of the normal incubation period, (2) before the end of the usual brooding period, and (3) during the feeding period.

#### AT THE END OF INCUBATION

To determine the effects of the presence or absence of young on the formation of the milk at the end of the incubation period, it was only necessary to have birds incubate infertile eggs. This opportunity was afforded in the castration experiments, where the birds incubated the infertile eggs. At the end of 18 days of brooding, the crops of all the females contained milk in spite of the fact that there were no young to excite the formation of the milk. It is evident,

therefore, that the formation of the milk proceeds even in the absence of the young.

### BEFORE THE END OF INCUBATION

Several tests were made by giving squabs from other parents to birds at different stages of incubation.

The evidence from these experiments is conclusive that the process of the formation of milk can be hastened very little, if at all, by the premature presence of young in the nest. These results corroborate the statement of Fulton (1895, p. 43), who observes on this point:

> We have seen how easily, in the case of most pigeons, the young ones may be "shifted" at almost any time desired within a fortnight; and pigeons will also readily take to and sit upon other eggs than their own; but it will *not* answer to give to any pair eggs partly hatched, unless laid at the same time as their own, and therefore due to hatch at the same date. The reason is obvious; the eggs hatching before the ordinary time of incubation is expired, there is *no soft food ready* for the young, and they must therefore perish. One day or perhaps two does not matter; but success when the shifted eggs have been sat upon more than this is very doubtful.

### DURING THE FEEDING PERIOD

The young are fed milk alone for the first 2 or 3 days. As they grow older, the milk is replaced partly with grain; after they are about 7 or 8 days old, grains are fed exclusively. If the feeding of milk alone for the first 2 or 3 days is a response to the age of the young, it should be possible to prolong the period of formation of the milk by substituting newly hatched squabs at regular intervals. The validity of this assumption was tested in several experiments, the results of which were negative in all cases.

Some light is thrown on the problem of the exclusion of grain from milk fed the young for the first 2 or 3 days by observations made on the condition of the crops of the parent birds during incubation and feeding of the young. From the seventeenth day of incubation to the fourth day of feeding, grain is not found in the crops of the parent birds, even just after they are fed. This is apparently due to the enormous hypertrophy of the epithelium, which greatly decreases the cavities of the pouches. The space which remains is occupied by milk, and thus no place is available for the storage of grain. From the fourth day of feeding onward, the processes of cell multi-

plication and desquamation begin to regress, and consequently more space is available for the collection of grain. It is apparent, therefore, that the omission of grain for the first 2 or 3 days and its subsequent inclusion in the feed of the young is not due to any special regulatory power possessed by the pigeons but is the direct result of the histological changes in the crop during this period.

## RELATION OF TESTES TO MILK FORMATION AND TO INCUBATION

### CASTRATION

Twenty-one males were castrated, some with complete success; in other cases the castration was incomplete. All but two of these males were already mated at the time of castration. The castrate period before the beginning of incubation was partly controlled by breaking up the reproductive cycle at the start of a previous brooding. Two males were castrated in the beginning of their first incubation.

### GROUP 1: CASTRATION COMPLETE

*Effects on the changes in the crop.*—In completely castrated males normal crop development was found at the end of the first incubation regardless of whether the operation was performed during the incubation period or at some time previous to it. The castrate periods before the commencement of the first incubation ranged from 3 to 16 days.

During the second and subsequent incubations after the operation the effects of the castration were evident. In all of these birds the histological picture of the crop was indistinguishable from that in Plate I, Figure 1, which represents the non-brooding stage. The castrate period before the beginning of the second incubation varied from 28 to 78 days.

*Effects on behavior.*—Changes in sexual behavior caused by prepuberal and postpuberal castration in other birds have been reviewed by Lipschütz (1924); and, as was to be expected, the results in the pigeon showed a more or less complete suppression of sexual activities. Incubation behavior did not disappear completely in all the castrated males, although there was a progressive decrease in its intensity and regularity. In the incubation period during which the

operation was performed, the brooding behavior and crop development remained normal; the male showed the same degree of stubbornness in leaving the nest as does an unmutilated male. During subsequent incubations, however, a mere approach to the pen sufficed to make the male leave the nest, and the crop changes did not occur.

Two males were unmated at the time of castration. Five weeks after the operation they were put with females but showed no interest in them and lacked the pugnacity so obvious in normal males placed with strange birds. The courtship behavior was entirely lacking. Nevertheless, in spite of the lack of courtship and mating, the females with these castrated males laid eggs several times. Kaufman (1932) obtained results consistent with those here reported.

The fact that the male would continue incubation, produce the milk, and feed the young after being castrated early in the period was also demonstrated by Riddle and Dykshorn (1932); but these investigators did not carry the experiment beyond the one period.

### GROUP 2: CASTRATION INCOMPLETE

Despite the fact that the amount of testicular tissue left in some males was exceedingly small, such males exhibited masculine behavior during courtship and normal crop development during incubations. No intermediate condition either in the growth of the crop or in behavior was noticed. In one case the testicular tissue found at autopsy weighed only 0.025 gm., which is about 1.4 per cent of the total testicular tissue in a normal male. Active spermatogenesis was the rule in the testicular nodules. There was, however, no appreciable increase in the amount of intertubular tissue.

Kaufman (1932) also mentions normal nesting behavior and crop development in an incompletely castrated male pigeon.

### MALE HORMONE

It seems apparent that the effects of castration, which prevents crop hypertrophy in the male during the second and subsequent incubations following the operation, are due, at least indirectly, to the absence of a testicular incretion. Attempts were accordingly made to rebuild the normal condition by injections of preparations of male hormone. The urine of young men was used, the method of extrac-

tion being that of Funk, Harrow, and Lejwa (1929, 1930). The potency of the extracts was tested by their effects on comb growth in capons.

Five castrated males were injected intraperitoneally three times a day with 0.5 cc. of urine extract (1 cc. = 200 cc. of urine) from the first day of incubation to the end of the incubation period subsequent to the first, but no crop development resulted. Consequently four males were injected three times a day with 0.5 cc. of a more concentrated extract (1 cc. = 400 cc. of urine). These results were also negative. The "dosages" employed varied from 3 to 6 cock units a day, which should eliminate the question of insufficiency of the hormone as an explanation for the negative results.

Two castrated males were injected intraperitoneally every other day during the incubation period with 1 cc. of blood serum from normal brooding males. The results were negative in both the birds.

In another experiment six testes from normal incubating males were implanted in two castrated males during the incubation periods of the latter. No positive effects were produced during the incubation in which the implantations were made, nor in the one following. At autopsy the implants were found to be resorbed.

Kaufman (1932) employed aqueous extract of pigeon testes on both brooding and non-brooding castrate males, also with negative results.

### CHANGES IN THE TESTES DURING INCUBATION

A correlation between the activity of the crop during incubation and the gonads was reported by Champy and Colle (1919). They report reduction in size of the testes during incubation reaching a maximum just before the hatching of the young, after which there is a gradual return to normal. Accompanying the reduction in size they reported a histological regression consisting of resorption of a large number of tubules and suppression of spermatogenesis in the remaining tubules. These changes, however, were unlike those observed in the winter season, for during incubation the interstitial tissue was absent in the testes, while it is abundant during the winter. In the female, during incubation, they observed an atresia of a large number of oöcytes; and this, as in the male, attained its maximum at the end of the period, thus corresponding in time with the maximum changes in the crop.

Our observations on the testes during incubation do not agree with those of Champy and Colle. The average weight of testicular tissue in six non-brooding males was 1.825 gm., and in six incubating males 1.816 gm. The difference is not significant. We have found neither a reduction in the number of tubules nor a suppression of spermatogenesis during incubation. This is evident in Plate II, Figure 11, which is a photograph of a section of a testis on the sixteenth day of incubation. The different stages of spermatogenesis are well marked.

## DISCUSSION

The experiments herein reported demonstrate clearly that the act of brooding has a definite relation to the normal development and functioning of the crop in the production of "milk." What part the sex glands play in the process is not quite so clear. Riddle and Braucher (1931) first showed that by the use of anterior pituitary extracts the crop development could be induced in male pigeons that had been castrated several months before. Later it was determined (Riddle, Bates, and Dykshorn, 1932, 1933) that the principle concerned is distinct from the already-known growth and sex-maturity hormones of the anterior pituitary, and it was accordingly designated "prolactin." Riddle and Dykshorn (1932) summarize their conclusions regarding the rôle of the testes as follows:

> There is no valid evidence that the testes play any direct part in the activation of the crop-glands. The testes do, of course, induce or influence the copulations which normally lead to the male's impulse to incubate; and a completed term of incubation apparently induces the anterior pituitary to release the specific hormone which in turn activates the crop-glands. The presence of this activator in the blood is as effective in the absence as in the presence of the testes.

Our results, as well as those of Kaufman, have shown that castrated males do not have the crop development in incubation periods beyond that following the operation, even though they may continue to incubate in relatively normal fashion; and it is to be presumed that the pituitary in this case is not producing the specific hormone for crop development. Riddle and Dykshorn are inclined to attribute this result, in a case earlier reported by Kaufman and Dabrowska (1931), to a lack of synchrony with the mate or to less intense incubation on the part of the castrated male. While this may

have been true in that particular case, there is room for considerable doubt as to whether it would apply to our cases or to the later ones reported by Kaufman (1932). Unfortunately, our records are not sufficiently complete to allow a positive statement as to the intensity of incubation by the castrated males that remained continuously with their mates—critical evidence would require almost continuous observation; but on the basis of observations made two or three times a day and oftener, they appeared to be carrying their regular share of the responsibility. The experiments on the temporary periodic removal of normal males demonstrated that the crop development is closely dependent on the length and continuity of incubation; but it is still possible that a more intense initial stimulus—such as copulation—is necessary to initiate the pituitary activity, and we have no evidence that this occurs in the case of the castrated males.

Another interpretation seems possible and has some evidence in its support. That the testis is necessary for males not yet mated to pair up is indicated by the behavior of two birds castrated before being mated, and which thereafter, though tried with females, would not mate. On the other hand, when males of mated pairs were castrated, they continued to respond to the incubation cycles of their mates even though the operation was 12–16 days prior to laying of the eggs. In these males, furthermore, the crop glands functioned in the first incubation period following the operation, but not thereafter. The difference between the first and subsequent incubation periods after operation may logically be attributed to the persistence of testicular influence for a period following the operation. Whether this influence is the direct action of a sex hormone, or whether previous mating has conditioned the nervous system so that mating behavior continues, cannot be decided on the basis of present facts. It would appear, however, that the production of prolactin by the anterior pituitary depends not alone on the psychic stimulation, but that there must either be sex hormone present at the time or the pituitary must previously have been conditioned by such a hormone. It is to be remembered that the prolactin used by Riddle and his associates to induce crop development in castrated pigeons came from pituitaries of animals (mammals) which presumably had developed it under the influence of active sex glands, at least of ovaries.

It would be interesting to determine whether the pituitaries of *castrated* mammals only would produce the same reaction.

The fact that the crop milk is formed by female as well as by male pigeons demonstrates that, whatever influence the sex gland has, it is not peculiar to the testis. This does not, however, rule out the possibility that the same hormone may be operating in the two sexes, since Kabak (1933) reports finding male hormone present in the urine of women in sufficient amounts to produce the typical comb responses in castrated cocks.

The alternative suggestions as to the chain of events leading to the crop hypertrophy here presented differ only in the rôle attributed to the gonad. This may be illustrated by the accompanying diagram:

$$
\begin{tikzcd}
\text{Gonad} \arrow[d] \arrow[r, dashed] & \text{Stimulation of pituitary} \arrow[r] & \text{Production of prolactin} \arrow[r] & \text{Crop response} \\
\text{Courtship} \arrow[r] & \text{Incubation} \arrow[u]
\end{tikzcd}
$$

The simple chain of events, as suggested by Riddle, would be represented by the diagram if the broken-line arrow is omitted. The gonad stimulates courtship, which is followed by incubation; this chain stimulates the pituitary to the production of prolactin, followed by crop enlargement. This view encounters the difficulty of explaining the failure of incubation to induce the pituitary action in castrated males which continue incubation through several cycles. The other suggestion is that the gonad is not only necessary for initiating normal sex behavior but it also influences the pituitary directly (or possibly by previous conditioning) to produce the crop-developing hormone. In connection with this view, however, it must be recalled that attempts to supply the male hormone in incubating castrated males did not lead to the crop response. Further investigation will be required to determine the relative rôles of the sex glands and of the psychosexual behavior.

## SUMMARY

1. The histological changes in the crop during incubation and feeding of the young were studied in closely graded stages. The observations confirm those of Litwer (1926) and Beams and Meyer

(1931) that the "pigeon's milk" consists of masses of the epithelial cells, loaded with fat globules, from the wall of the lateral pouches of the crop of the parent birds. The histological changes leading to the formation of the milk are gradual and consist in an increased cell multiplication in the stratum germinativum, followed by desquamation of the surface cells of the thickened epithelium.

2. If the male of a mated pair is put in a separate cage adjacent to and whence he can see his incubating mate, his crop has the normal development at the end of 18 days, and he is capable of feeding the young. This influence, in the absence of actual brooding, is referred to as "psychological brooding."

3. Removal of the male from sight of the female, whether close by or in another room, prevents the progress of changes in his crop. Under these conditions the psychological brooding does not occur.

4. The changes in the crop of the male can be checked and started again several times during the same incubation period by alternating the normal brooding with periods during which he is prevented from sitting on the eggs and is also removed from sight of the incubating mate.

5. As a result of the long intervals of the lack of both actual and psychological brooding, interrupted by the short periods of normal brooding, the changes in the crop of the male do not attain their normal condition within the usual incubation period. Normal crop development may, however, be secured by prolonging the incubation beyond 18 days.

6. Formation of milk occurs at the end of 18 days of incubation even in the absence of squabs.

7. The substitution of young for eggs early in the incubation period does not provoke the formation of milk before the normal time.

8. It was found impossible to prolong the period of milk formation by substituting newly hatched squabs successively during the feeding period.

9. The exclusion of grain from the food of the young for the first 2 or 3 days is attributed to the great thickening of the crop of the parents, which leaves no space for the storage of grain.

10. When young do not supervene at the normal hatching time, milk formation continues as long as the birds incubate the eggs beyond 18 days.

11. Castrated males that were mated at the time of castration produce "milk" normally during the first following incubation period but not in succeeding ones.

12. If castration is incomplete, crop hypertrophy is normal in all succeeding incubations even though the piece of testicular material remaining is very minute.

13. Extract of male urine, blood serum from brooding male birds, and implants of testis into inactive castrates failed to produce the crop response.

14. The observation of Champy and Colle that there is an involution and decrease in size of the testis coincident with the increasing hypertrophy of the crop was not confirmed.

15. While it is possible that the gonads influence milk formation only indirectly through their effect on sexual behavior, it is still an open question whether they may not also have a direct effect on the pituitary activity.

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# THE OXYGEN CONSUMPTION OF HEXAGENIA RECURVATA DURING THE WINTER AND EARLY SPRING

(Three figures)

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LIKE other May flies, the large burrower, *Hexagenia recurvata*, lives in the water during its immature or nymphal stage, which in this species lasts for about two years. These nymphs live in ponds and slow streams, tunneling their way through the mud or creeping over the bottoms. Where they occur at all, they are usually abundant and obtainable throughout the winter in goodly numbers. The nymphs used in these experiments were taken from one region of a spring-fed brook near South Hadley, Massachusetts, where *H. recurvata* nymphs were also collected for an earlier study of the functions of their gills (Morgan and Grierson, 1932).

## MATERIAL AND METHODS

*Material.*—Eighteen collecting trips were made to Lithia Springs Brook during the fall and winter (September 19, 1934—March 15, 1935) and one trip on May 3. Observations of weather conditions, temperature of the water, and activities and abundance of the nymphs were made on each trip. Nineteen hundred and eighty-five nymphs, ranging from 10 to 30 mm. in length, were collected.

Immediately after the field trips the nymphs were measured and caged in groups according to size. Two kinds of cages were used. The stock cages were 1 foot in diameter and had sides and bottom of copper-wire screen. These were set in galvanized iron trays 2 inches deep, each having a capacity of 150 nymphs. The small cages holding from one to seven nymphs were used for those which had been chosen for observation. Each of these was a 3×4-inch compartment separated off by wire screening in a multiple-unit tray of forty such cages. All cages were supplied with running water and diatom-saturated ooze from the nymphs' own habitat. Fresh mud was

added from time to time, and in February filamentous algae were stirred in with it.

About once a week the small cages were examined for molted skins and dead nymphs. The stock cages were examined once a month. Four other cylindrical screen cages, containing a total of about twenty nymphs, were kept in Lithia Springs Brook as a check on the condition of the laboratory-kept nymphs.

During the spring and fall the death-rate was high. Through the winter, however, probably as a result of the lowered metabolism accompanying winter conditions, the death-rate was lowered to about one out of eight nymphs per week.

*Organization of the experiments.*—Twelve experiments were conducted from October 27 to March 17, but the first three of these were discarded because of faults in technique. Of the remaining nine, three were performed in December (December 1, 15, 29), three in January (January 2, 12, 29), two in February (February 9, 23), and one in March (March 16).

Each experiment was set up as follows: three 500-cc. bottles each containing 250 cc. of distilled water and three nymphs and one bottle with the same water content but no nymphs (the blank) were set in each of three water baths, whose average temperature was respectively 1.0°, 6.4°, and 12.3° C. Thus, twelve bottles were used. Of the nine containing nymphs, usually two out of the three in each temperature held recently collected nymphs, while the others held nymphs that had been kept in the laboratory from 10 days to 2 months.

The experiments lasted 25 hours. Five determinations of the oxygen content of the water in the bottles were made, namely, at the start and at the fifth, tenth, fifteenth, and twenty-fifth hours.

*Care of nymphs before the experiments.*—After the nymphs had been brought to the laboratory, they were at once sorted and grouped according to size. Those which were to be kept in the laboratory were placed either in the stock or unit cages before described. The largest nymphs available (approximately 27 mm.) were used in the experiments. Sometimes these could not be secured and it was necessary to use 24–25-mm. lengths. They were acclimated by being placed in bottles set in water baths maintained at the temperatures

at which the tests were to be made. It was hoped that the period of acclimatization would provide time for the digestion of food already consumed, as well as accustom the nymphs to the temperature at which they were to be tested. The 500-cc. bottles used for acclimatization were the same ones in which the nymphs were later tested. Wire mesh was placed in the bottom of each bottle as a foothold for the nymphs.

*Maintenance of constant temperature.*—The lowest temperature, 1° C. used in the experiments was obtained by the use of an electrically controlled constant-temperature bath. The medium temperature was maintained by an even flow of water into a museum jar containing the experimental bottles. This temperature remained constant ±1° through an experiment, but varied from month to month, showing a slow decline from 7.0° C. on December 1 to 5° C. on March 15, with a seasonal average of 6.4° C. The highest average temperature, 12.3° C. (average of the highest temperatures in each of nine experiments) was obtained by running tap water through a 1-liter Erlenmeyer flask heated by the coils of an electric unit. After some experimentation this temperature could be kept constant ±3° during an experiment, but it varied slightly from experiment to experiment.

*Oxygen determinations.*—Following the practice of Hiestand (1931), during experimentation all nymphs were kept in distilled water, which appeared to have no harmful effect upon them. Since the determinations were made by the unmodified Winkler method, it was thought that by the use of the distilled water errors due to impurities in the water could be avoided. The distilled water was brought to the right temperature and, just before using, was shaken up with air in order to oxygenate it.

The experiments were started in the morning following a collection of the nymphs. Bottle A, containing nymphs collected the day before and acclimated at 1.0° C. through the night, was taken from the water bath. The acclimatization water was poured out. The bottle was then rinsed with distilled water of the same temperature, and finally 250 cc. of distilled water was siphoned into the bottle. A deep layer of motor oil was then poured onto the water. The cork was pressed in and oil added through a small hole in the cork till the

bottle was completely full. The hole was then plugged, and the whole top coated with melted paraffin. The bottle was then completely submerged in the water bath. In the earlier experiments the glass bend of the exit siphon was left above water, but bubbles of gas collected there; so in later experiments it was submerged. Fifteen minutes after the procedure with bottle A, the same thing was repeated for bottle B; 15 minutes after that, for C; and so on. Thus, each of the twelve bottles was started in sequence 15 minutes apart. The determinations were made at the fifth, tenth, fifteenth, and twenty-fifth hours and each took 15 minutes to perform; thus the contents of each bottle could "run" the same length of time. Before each experiment determinations were made of the oxygen content of the water to be used in the test bottles.

The determinations were made in a Thompson-Miller apparatus for the micro-Winkler method, 5.5 cc. capacity. Before each test this was filled to capacity with water from the experimental bottle, then emptied, and refilled to capacity for the test. Thus, equal amounts of water were always removed from all bottles in each series of determinations. After each test the apparatus was rinsed, first with tap water and then with distilled water. Directions for the use of the apparatus are given by Thompson and Miller (1928); and notes on its accuracy, by Snoke (1929) and Allee and Oesting (1934). As soon as the sample had been drawn from the bottle, more oil was added in order to bring the oil surface to the level of the cork again. The top was then resealed with paraffin. The condition of the nymphs was always noted during these operations and before the bottle was replaced in the bath.

The water sample was treated according to the method of Kremmerer, Bovard, and Boorman (1923) and modified to suit the Thompson-Miller micro-apparatus. The sodium thiosulphate was standardized against N/100 potassium permanganate instead of against potassium bichromate. The equation used was that given by these workers for finding the amount of oxygen in cubic centimeters per liter. This was changed to milligrams, and the milligrams per gram body weight computed.

*The use of motor oil as a seal.*—Motor oil was used to cover the water during the tests in all the experiments. It was finally adopted

after considerable experimentation and practice. In preliminary experiments the water was covered with paraffin oil (Amberson, Mayerson, and Scott, 1924; Morgan and O'Neil, 1931; and others). It is well known that atmospheric oxygen will penetrate through paraffin oil, especially after periods longer than 10 hours. These experiments on *Hexagenia* lasted 25 hours. The 500-cc. bottles were chosen in order to allow for a deep layer of paraffin oil over the water. Even with this precaution some leakage was detected by a rise of oxygen in the water of the blank bottles.

By the use of luminescent bacteria Hill (1928) showed that oxygen would go through paraffin oil and into water within 6 hours but that it would not go through motor oil. In the practice experiments with the motor oil it was discovered that with this oil oxygen passed from the water into the oil. There was an apparently greater oxygen consumption by the nymphs in water covered by motor oil than there had been by those in water covered by paraffin oil, and the oxygen content of the blank bottles fell in proportion to the temperature. No deduction for the error inherent in the motor oil was made in the calculations of the oxygen consumption. However, the average loss from the water into the oil at three temperatures was measured and is presented in Table I.

In other experiments in this laboratory paraffin oil has served as well as motor oil when the test bottles were totally submerged and duplicate determinations averaged. Either motor or paraffin oil gave only comparative results. These, however, are satisfactory for the biological questions at hand. For absolute measurements, bottles should be sealed with mercury or inclosed in a nitrogen chamber.

## OBSERVATIONS

At a given temperature the average oxygen consumption of *Hexagenia* nymphs was nearly the same through the four succeeding 5-hour intervals from one determination to another (Table II). But variation in the rate of consumption occurred in individual sets of animals. Such variation of individual oxygen consumption from hour to hour is not uncommon amongst invertebrates (Dakin and Dakin, 1925, p. 315).

At the lower temperatures 1.0° C. and 6.4° C. the consumption of the nymphs was unaffected by the decreasing oxygen tension. At

TABLE I

AMOUNTS OF OXYGEN THAT ESCAPED FROM DISTILLED WATER INTO A SEAL OF MOTOR OIL IN EXPOSURES MADE AT THREE TEMPERATURES

Amounts given are the averages of determinations made on the "blank bottles" of five experiments (January 1 through March 16), given in milligrams of oxygen per liter of water.

| Temperature Degrees C. | Oxygen Content at Start | Oxygen Loss at— | | | |
|---|---|---|---|---|---|
| | | 5 Hours | 10 Hours | 15 Hours | 25 Hours |
| 1.0 | 9 603 | 0.552 | 0.737 | 1.313 | 2.189 |
| 5 64 | 8.619 | 0.476 | 0.745 | 1.465 | 2.356 |
| 11.88 | 7.491 | 1.349 | 2.158 | 2.856 | 4.130 |

TABLE II

THE COMPARATIVE OXYGEN CONSUMPTION OF RECENTLY COLLECTED (REC. COL.) AND LABORATORY-KEPT (LAB. KEPT) NYMPHS OF *Hexagenia recurvata* DURING 5, 10, 15, AND 25 HOURS AT THREE TEMPERATURES

Average of twenty-fifth hour determinations at 12.3° C. based on January–March only. An average of nine experiments. Amounts of oxygen in milligrams per gram body weight.

| Hours of Consumption | Nymphs | 1.0° C. | 6.4° C. | 12.3° C |
|---|---|---|---|---|
| 5 | Rec. col. | 2.305 | 2.982 | 5.510 |
| | Lab. kept | 1.444 | 2.503 | 4.460 |
| 10 | Rec. col. | 3.582 | 6.058 | 9.387 |
| | Lab. kept | 1.960 | 4.592 | 8.229 |
| 15 | Rec. col. | 5.006 | 8.598 | 12.174 |
| | Lab. kept | 3.359 | 6.885 | 11.558 |
| 25 | Rec. col. | 7.584 | 11.950 | 8.66 |
| | Lab. kept | 5.203 | 10.692 | 7.89 |

12.3° C. the consumption decreased between the fifteenth and twenty-fifth hour, but this was probably due to the effect of accumulating carbon dioxide.

Singh-Pruthi (1927) found that May-fly nymphs could remain in water with oxygen tensions as low as 0.3–0.2 cc. per liter without ap-

parently being affected, provided the pH and the tension of carbon dioxide were right. Many *Hexagenia recurvata* nymphs died after 15 hours at 12.3° C., and it is believed that carbon dioxide narcosis may have been responsible.

*The oxygen consumption of recently collected and laboratory-kept nymphs.*—The average oxygen consumption of nymphs kept in the laboratory 10 days or more is less than that of nymphs collected within 24 hours.

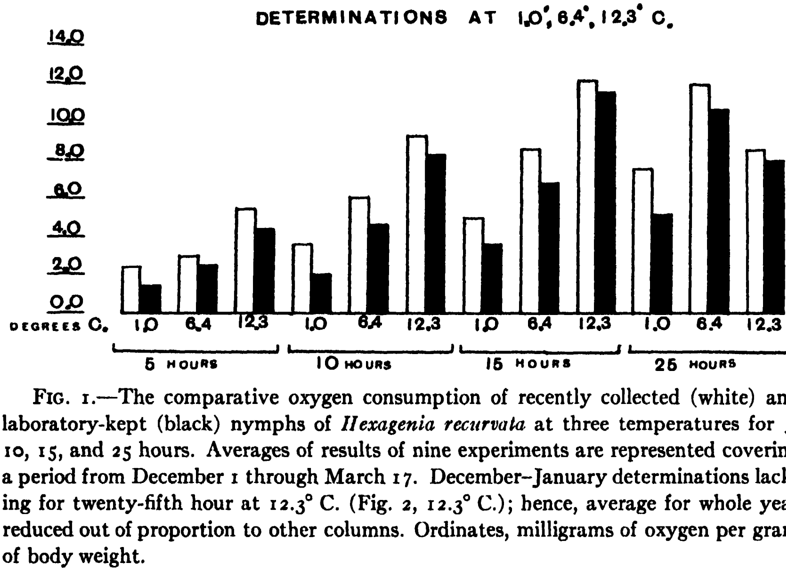


FIG. 1.—The comparative oxygen consumption of recently collected (white) and laboratory-kept (black) nymphs of *Hexagenia recurvata* at three temperatures for 5, 10, 15, and 25 hours. Averages of results of nine experiments are represented covering a period from December 1 through March 17. December–January determinations lacking for twenty-fifth hour at 12.3° C. (Fig. 2, 12.3° C.); hence, average for whole year reduced out of proportion to other columns. Ordinates, milligrams of oxygen per gram of body weight.

Averages of the results of nine experiments each involving an average of 22.8 nymphs revealed that laboratory-kept nymphs showed a consistently lower oxygen consumption than recently collected ones (Table II and Fig. 1).

The exception indicated in Figure 2 in the fifteenth-hour determinations (December 1—January 13) at 12.3° C. is only an apparent one. Several of the recently collected nymphs involved in these 12.3° C. tests showed a lessened resistance toward the end of the experiments, which allowed the laboratory-kept nymphs to overtake them in oxygen consumption. In several cases the nymphs died by the fifteenth hour. From December 1 to January 13 no consumption record was used for the twenty-fifth hour at 12.3° C., because one or

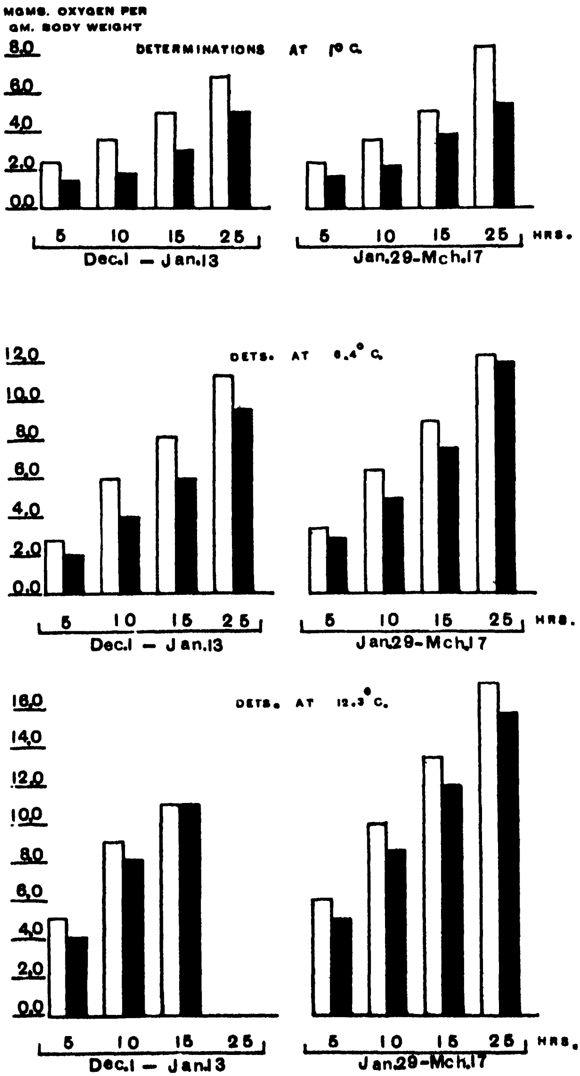


FIG. 2.—The average oxygen consumption of recently collected (white) and laboratory-kept (black) nymphs of *Hexagenia recurvata*, showing (1) the early-winter period of low oxygen consumption followed by the late-winter and spring rise, and (2) the higher consumption of the recently collected nymphs (for explanation of exception in fifteenth-hour determination at 12.3° C. see p. 159). Abscissa, intervals at which determinations were taken; ordinate, milligrams of oxygen per gram of body weight. Averages based on five experiments for the first and four for the second periods.

two out of the three nymphs were dead before this hour of this series. Such records were discounted (twenty-fifth hour, 12.3°, Table III).

TABLE III

THE AVERAGE OXYGEN CONSUMPTION OF RECENTLY COLLECTED AND LABORATORY-KEPT NYMPHS OF *Hexagenia recurvata* FOR THE EARLY-WINTER (DECEMBER 1—JANUARY 13) AND THE LATE-WINTER PERIOD (JANUARY 29—MARCH 17)

Tests made at three temperatures. Amounts of oxygen in milligrams per gram of body weight. Average based on nine experiments each involving an average of 22.8 nymphs.

| Hours of Consumption | Season | 1.0° C. | 6.4° C. | 12.3° C. |
|---|---|---|---|---|
| | Recently collected nymphs | | | |
| 5 | Dec.–Jan. | 2.265 | 2.260 | 4.998 |
| | Jan.–March | 2.346 | 3.305 | 6.021 |
| 10 | Dec.–Jan. | 3.508 | 5.774 | 8.927 |
| | Jan.–March | 3.657 | 6.343 | 9.847 |
| 15 | Dec.–Jan. | 4.948 | 8.241 | 10.936 |
| | Jan.–March | 5.063 | 8.955 | 13.411 |
| 25 | Dec.–Jan. | 6.805 | 11.479 | ............ |
| | Jan.–March | 8.362 | 12.420 | 17.327 |
| | Laboratory-kept nymphs | | | |
| 5 | Dec.–Jan. | 1.304 | 2 079 | 3.970 |
| | Jan.–March | 1.585 | 2.927 | 4.950 |
| 10 | Dec.–Jan. | 1.709 | 4.024 | 7.804 |
| | Jan.–March | 2.210 | 5.160 | 8.654 |
| 15 | Dec.–Jan. | 2.944 | 6.036 | 10.995 |
| | Jan.–March | 3.774 | 7.734 | 12.122 |
| 25 | Dec.–Jan. | 5.016 | 9.503 | ............ |
| | Jan.–March | 5.390 | 11.801 | 15.784 |

Correlated with the higher metabolism indicated by the respiration of the recently collected nymphs was the fact that between March 15 and May 3, 78.4 per cent of them had molted, while between April 15 and April 29 only 5.3 per cent of the laboratory-kept nymphs had done so.

As evidenced by the oxygen consumption just mentioned, it appears that the metabolism of the nymphs was lowered by laboratory conditions, even though the temperature of the water in which they were kept was approximately 5° higher than that of their own habitat. This emphasizes the fact that in physiological studies it cannot be taken for granted that animals are living at their normal rate. Dreyer (1932) calls attention to the importance of the imitation of natural conditions for animals that are being used for physiological experiments. He was able to imitate the ant's normal habitat and found that the respiratory behavior of laboratory colonies was "similar to that of animals brought directly from the field nests." The laboratory conditions in which *Hexagenia recurvata* lived were certainly as good as those of most aquatic animals in the laboratory. These nymphs were in constantly flowing water of high oxygen content and a pH of 6.8, only two-tenths less than that of their own habitat, Lithia Springs Brook. Lack of space seems unlikely to have hurt them, since a group of fifteen nymphs tried out in a $3 \times 4$-inch cage apparently lived as well as did one to five nymphs in cages of the same size. Metabolic products could not have been an influence, since currents of water constantly cleared these away. However, there were obviously unavoidable differences between the laboratory and native environments, such as those incident to depth of water and to materials in solution.

*Oxygen consumption as affected by season.*—The oxygen consumption of all nymphs was higher in February and March than it had been earlier in the winter. When the consumptions in five early winter experiments (December 1 through January 3) were averaged and compared with the four late winter ones (January 29 through March 17), the results showed that the late ones were greater in every case (Table III and Fig. 2).

This seasonal rise involves the problem in much greater complexity. If the metabolism of the nymphs were dependent upon the temperature of the environment, as Dreyer (1932) has found for the ant, a constant temperature would lead one to expect a steadily maintained metabolism, and a falling temperature a lowered metabolism. But the rise in oxygen consumption was not correlated with a rise in habitat temperature in either freshly collected or in labora-

tory-kept nymphs, as records of habitat temperature and oxygen consumption show (Fig. 3, graphs *A*, *B*, and *C*). The January rise

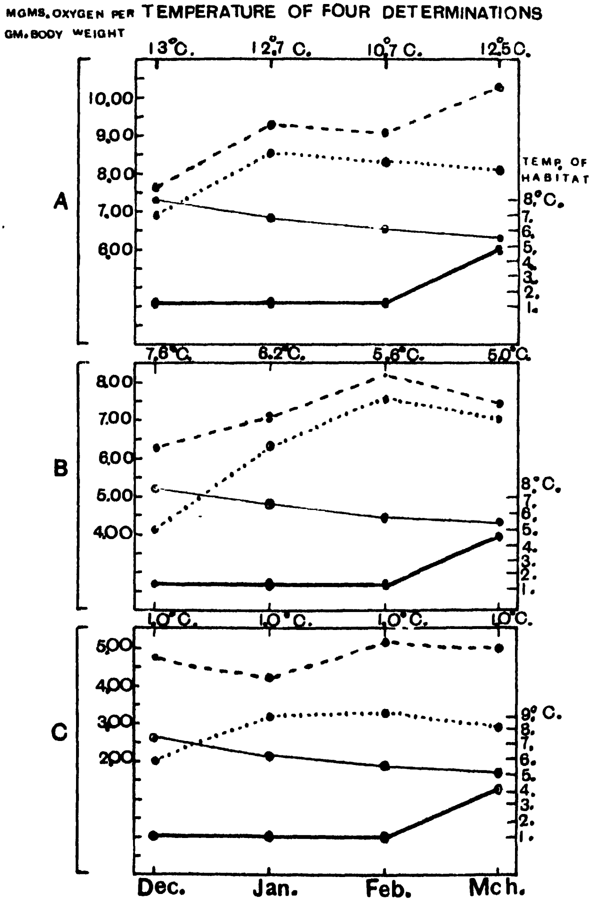


FIG. 3.—The average oxygen consumption of recently collected (heavy dash line) and laboratory-kept (light dot-and-dash line) nymphs of *Hexagenia recurvata*, December 1 through March 17, showing monthly fluctuations and the temperature of their habitats: temperature of native stream (heavy continuous line), temperature of tap water (light continuous line). Abscissa, months when determinations were made; ordinate, milligrams of oxygen per gram of body weight.

occurred, without exception, in both types of nymphs, except that in those tested at 1.0° C. it was not found until February (Fig. 3, graph *C*):

In the laboratory-kept nymphs and in the recently collected ones tested at a temperature higher than that at which they had been living the late-winter rise was first noticed in January (Fig. 3, graph *A*). In recently collected nymphs tested at the temperature of their habitat (1.0° C.) the rise occurred in February (Fig. 3, graph *C*). None of these rises can be correlated with temperature, since in Lithia Springs Brook the temperature was about the same as on earlier winter dates (December, 1.5°; January, 1°; February, 1° C.) and in water at the laboratory it was lower (Fig. 3, graphs *A*, *B*, *C*, temperature of habitat). A correlation of increased body weight and proportionately lowered oxygen consumption was observed in the March determinations.

The fall in the March consumption of recently collected nymphs shown in Figure 3, graph *B*, may be correlated with the fact that these nymphs were the heaviest ones collected. In individual experiments it was found that a heavy nymph consumed proportionately less oxygen than a lighter one. The converse is shown in graph *A* of the same figure, where the March rise is in the oxygen consumption of lighter-weight nymphs. At this season the greater growth activity of these smaller nymphs created a greater oxygen demand, in addition to that resulting from the greater proportion of surface to the volume of their bodies.

Contrary to these observations, Dreyer (1932), as already mentioned, found that the oxygen consumption and respiratory exchange in the ant paralleled the temperature, whether natural or experimental. He kept ants, *Formica ulkei*, in a hibernating condition throughout the year by placing them in a low temperature, and in an active condition by keeping them in a high temperature, and determined that their respiratory exchange closely followed their surrounding temperature throughout the year.

He has thus shown that the rate of metabolism in the ant is generally dependent upon environmental temperature. When the temperature was varied experimentally, the oxygen consumption of nymphs of *Hexagenia recurvata* was also affected by the temperature (Tables II and III; Fig. 1). On the other hand, in January and February their oxygen consumption rose in spite of consistently low temperatures (Fig. 3). Thus it becomes apparent that, while these

nymphs may be affected experimentally by temperature, their metabolism is nevertheless, to a considerable degree, independent of it. It is likely that there is a similar situation in the winter stone flies, as their winter behavior demonstrates. *Capnia* and *Taeniopteryx*, which are active, grow rapidly and even emerge into the air as adults as early as January and February when the temperature of their habitat is as low or lower than it has been earlier in the winter.

*The oxygen consumption as affected by experimentally varied temperature.*—In these experiments, as already stated, three temperatures (1.0°, 6.4°, and 12.3° C.) were used, and three levels of oxygen consumption resulted (Tables II and III, and Figs. 1 and 2). The objection may be raised that the absorption of oxygen by the motor oil might account for these levels. This objection has been considered. The loss of oxygen from the water into a seal of motor oil was determined for three temperatures at 5-, 10-, 15-, and 25-hour intervals (Table I). The loss of oxygen in the oil at 25 hours at 1.0° C. was, on the average, 2.189 mg. of oxygen per liter of water; at 5.6° C. it was 2.356 mg.; and at 11.88° C. it was 4.130 mg. The increase in loss of oxygen between 1.0° C. and 5.6° C. is not proportionate to the much greater loss, owing to its consumption by the nymphs (cf. Tables I and II). With the experimentally varied temperatures the average increase in oxygen consumption with a rise of 6° was 1.26 times that at 1.0° C.; and with a rise of 12°, 2.77 times that at 1.0° C. When these figures were calculated on the basis of the $Q_{10}$ of van't Hoff (Rogers, 1929), a $Q_{10}$ value of 3.319 was obtained from the temperature rise of 1.0°–6.4° C., and 2.645 from the rise of 6.4°–12.3° C. The smaller $Q_{10}$ for the rise from 6.4° to 12.3° C. indicates that at the higher temperatures factors such as carbon dioxide accumulation (Fig. 2, December 1—January 13, 15-hour determination, and death of nymphs before 25 hours) were probably operating on the nymphs to reduce their metabolism.

## DISCUSSION

The fact that *Hexagenia recurvata* consumed less oxygen during the first part of the winter indicates its depressed metabolism in that season. Possibly in November, certainly in December and the first

part of January, occurs its winter "low." Near the end of January, and in February and March, there is a definite rise in oxygen consumption, evidently correlated with the quickened growth and activity characteristic of May flies at this season.

Does other behavior of the nymphs in any way express their "low" winter condition? When its light response changes from positive to negative, the metabolism of the May-fly nymph *Epeorus* is lowered (Allee and Stein, 1918). In a series of preliminary tests made upon the light responses of the *Hexagenia recurvata* nymphs under various conditions, there was some evidence that, on the average, they were more negative to light in October and February than in March and May. A possible correlation is thus indicated between their phototropism and respiratory metabolism. A relationship of this kind has been suggested by Allee and Stein (1918) in nymphs of the May flies *Epeorus* and *Leptophlebia*. Using potassium cyanide as an index of metabolism, they demonstrated a correlation between phototactic reaction and metabolic condition. In their normal behavior Holmquist (1928) found that, in the cold, muscid flies were inactive and had a negative reaction to light; whereas in the warmth, they were more active and were positive to light. So far as they have been studied here, the responses of *Hexagenia recurvata* nymphs also indicate that they manifest their lower metabolism by more negative reactions to light during the winter and their higher metabolism by more positive ones in the spring.

Absence of molting may be taken as a manifestation of low metabolism in nymphs of *Hexagenia recurvata*, as it has been shown to be in the dragon fly, *Anax junius*, by Calvert (1929) and as discussed for insects by Singh-Pruthi (1925). No nymphs molted in the laboratory cages from December 3 to April 29. Neither were there any recently molted nymphs among those collected from Lithia Springs Brook between November 15 and March 15. On the other hand, of those taken on May 3, 17 per cent were recently molted ones. The oxygen consumption of the nymphs showed that their metabolism started to rise in the latter part of January. Its continued rise is indicated by the nymphs' greater positiveness to light in March and the frequent appearance of molted skins after April 29.

All nymphs of *Hexagenia recurvata* showed greater resistance to laboratory conditions between November and February, their period of lowered oxygen consumption, than they did before and after those months. In the examinations of the cages from November 12 to January 8, 4.96 was the highest percentage of nymphs found dead. But beginning on January 21 the mortality rose from 11.45 per cent to 38.15 per cent on April 15.

Experiments and natural behavior have shown that sluggish animals can endure an environment which would be fatal to them if their metabolism were more rapid. For example, animals with a higher metabolism are more susceptible to a strong solution of potassium cyanide than those with a lower one (Allee and Stein, 1918; Child, 1919; Hyman, 1919). It seems therefore that in *Hexagenia recurvata* the earlier resistance, and later lack of resistance, to unfavorable conditions of the laboratory is also a manifestation of their lowered metabolism during the winter and its rise toward spring.

It has already been shown that there is a metabolic rhythm in these nymphs which is, to a considerable degree, independent of temperature. Annual metabolic cycles are more evident in other insects undergoing a diapause, a period of low metabolism resulting in complete quiescence occurring independently of temperature. Nymphs of *Hexagenia recurvata* have no diapause, nor can they be said to be intermittently inactive. Yet, their period of low oxygen consumption, even though occurring during their winter of more or less continuous activity, is suggestive of the more striking hibernation phases of other insects.

## SUMMARY

1. Nineteen hundred and eighty-five nymphs of *Hexagenia recurvata* were collected from a spring-fed brook in eighteen collections, made mainly in the winter and early spring (September 19 to May 3). Observations were made upon their behavior in their habitat and under laboratory conditions in which they were kept from 10 days to 2 months.

2. Experimental tests of the oxygen consumption of a total of 205 laboratory-kept and recently collected nymphs were conducted from

October 27 through March 17. Three tests made in October and November were counted as preliminary; and the results of nine, performed in December, January, February, and March, are reported here.

3. The oxygen consumption of nymphs kept in the laboratory from 10 days to 2 months was less than that of nymphs collected from their own habitat and tested within 24 hours.

4. Between December 1 and January 13 the oxygen consumption of all nymphs was lowered; between January 29 and March 17 it rose. The nymphs thus exhibit a late-winter rise in oxygen consumption.

5. In all laboratory-kept nymphs and in recently collected ones tested at temperatures higher than that of their own habitat, a "pickup" in oxygen consumption occurred in the last part of January. In recently collected nymphs tested at the temperature of their own habitat, it occurred in February.

6. In experimentally varied temperatures the oxygen consumption was directly proportionate to the temperature, a $Q_{10}$ of from 2.6 to 3.3 being obtained.

7. In their rate of oxygen consumption nymphs of *Hexagenia recurvata* are independent of the oxygen tension from 9.75 to 2.34 cc. of oxygen per liter of water, despite accumulating carbon dioxide. They may survive when there is but 0.50 cc. of oxygen per liter of water.

8. Among other evidences of low metabolism was an absence of molting from December 3 to the end of April. Molting was frequent, however, during May and October.

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# THE EFFECTS OF CARBON DIOXIDE, THE HYDROGEN ION, CALCIUM, AND EXPERIMENTAL CONDITIONING ON RECONSTITUTION IN EUPLANARIA DOROTOCEPHALA.[1]

(Six figures)

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THE effects of acids on physiological processes have long been of interest to investigators. One has but to look at the tremendous amount of literature that has accumulated on the subject to realize that the H-ion is of prime importance. It is no doubt true that many biological phenomena have been attributed directly to the external H-ion when in reality the relationship may be very indirect. Alterations of the H-ion concentration may cause an increase or a decrease in the solubility of other substances in the solution, and this factor alone may be responsible for many phenomena that have been stated as being results of the direct action of the H-ion.

The effects of external $CO_2$ have often been confused with the effects of the external H-ion. The alteration of physiological processes of organisms in natural waters to which acid has been added has often been stated as being the result of H-ion rather than of the $CO_2$ which may have been liberated on acidifying the water.

In general it has been found that increased H-ion and $CO_2$ concentration in the external medium serve as inhibitors to biological processes. In recent years much work has been done in altering reconstitutional and embryological development through the use of various inhibiting agents applied externally. It has been found, in general, that agents which tend to inhibit development in organisms do so differentially. In other words, the more active regions may be inhibited to a greater extent than the less active regions. Certain phenomena of twinning, exogastrulation, and various other teratological processes are evidently examples of differential inhibition.

[1] The author wishes to thank Professor C. M. Child for much valuable advice and criticism received throughout the progress of this work.

In this investigation we are concerned chiefly with the effects of the H-ion and $CO_2$ on reconstitutional development.

### THE PROBLEM

Child (1911*a*) pointed out that transverse pieces of *Euplanaria dorotocephala*, of a limited size and from the more posterior levels of the first zooid, reconstituted in a large number of cases into headless individuals or individuals which showed various degrees of head inhibition. The frequency of inhibited head-forms and the degree of inhibition were found to be related to the level from which the piece was taken and to its length. Short pieces and pieces from the posterior levels of the anterior zooid showed the greatest inhibition in head-form. Child has placed the reconstituted head-forms in the following classes, corresponding to the degree of inhibition:

*Normal.*—Head triangular with two symmetrically placed eyespots and with auricles at lateral margins.

*Teratophthalmic.*—Head normal in shape and with auricles in normal position, but with eyespots showing all degrees of approximation to the median plane from two distinct eyespots with pigment connected to complete cyclopia.

*Teratomorphic.*—Head more or less rounded in outline with single or apparently single median eye; auricles more or less anterior and showing all degrees of approximation to the median plane up to a single median auricle.

*Anophthalmic.*—Heads rudimentary without eyespots and with or without a single median auricle.

*Acephalic.*—Head completely absent.[2]

It subsequently has been found and sustained by Child and others that the inhibition of normal head formation in transverse pieces of *Euplanaria* is related to some inhibitory influence which arises from the posterior cut surface of the isolated piece. This factor inhibiting head development is most effective during the first 24 hours after section; yet, it may still be evidenced, as shown by delay of anterior

[2] Since Child (1911*a*, 1911*b*, 1911*c*, 1912, 1914*a*, 1914*b*, 1916, 1920, 1924), Child and Watanabe (1935), and others (Behre, 1918; Buchanan, 1922; Hinrichs, 1924) have repeatedly described and figured the different head types in *Euplanaria*, it does not seem necessary to present either drawings or photographs of them here.

section, after 3 or 4 days (Child and Watanabe, 1935). It is quite evident that this inhibiting action of the posterior cut surface is not entirely the result of stimulation by section, since this stimulation, as indicated by increased respiration and susceptibility, disappears within a few hours (Child, 1914*a*; Robbins and Child, 1920; Hyman, 1923). It is probable that the cellular activity associated with the early stages of tail development also plays a part in the inhibition. The inhibiting factor is apparently largely or wholly nervous in character and can be decreased or obliterated by delay of posterior or anterior section or by section under anesthesia (Child, 1914*b*; Buchanan, 1922; Child and Watanabe, 1935; Watanabe, 1935*b*).

In dealing with the various types of heads that arise from lots of reconstituted worms, the term "head frequency" has been used. By head frequency is meant the frequency of the various forms that reconstitute from lots consisting of pieces of a certain length and from a certain body-level of animals of the same length. If a certain lot of pieces gives a preponderance of normal and near-normal animals, we speak of the lot as having a high head frequency. If a lot gives a majority of headless or strongly inhibited head-forms, we speak of it as having a low head frequency.

Many workers have found that pieces of planarians, which would normally give a low head frequency, gave an increased head frequency when allowed to reconstitute in certain slightly toxic solutions. Reagents which have been used to increase head frequency in the posterior levels of the first zooid are dilute KCN (Child, 1916), chloretone, chloroform, chloral hydrate, ether, ethyl alcohol (Buchanan, 1922), and caffeine (Hinrichs, 1924). Mechanical stimulation (Child, 1920) and abrupt change in temperature from 20° to 29° C. (Behre, 1918) have also been found to increase head frequency.

Child (1911*a*, 1912, 1916) has shown that the head frequency of pieces from anterior levels and of long pieces could be decreased by lowering the temperature or by allowing the pieces to reconstitute in dilute solutions of metabolic end-products, ethyl alcohol or KCN, for varying intervals of time. Behre (1918) found that animals, taken from a living temperature of 20° C., sectioned, and allowed to reconstitute in a temperature of 10° C., gave a decrease in head frequency. Buchanan (1922) found a slight decrease in the head frequency in

anterior pieces with short time exposure to anesthetics. Hinrichs (1924) found that anterior pieces showed a decreased head frequency with initial exposure of 24 hours to M/200 caffeine.

In general it would seem that those agents which increase head frequency do so either by inhibiting the effect from the posterior cut surface or by stimulating the head-forming cells at the anterior cut surface. A decrease in head frequency appears to be the result of an increased effect of the inhibiting factor or a decrease in the activity of the head-forming cells at the anterior cut surface.

Since the H-ion and $CO_2$ have been regarded as important factors in many biological processes, it was suggested that the reconstitution of *Euplanaria dorotocephala* in different concentrations of H-ion and $CO_2$ might well be investigated. Experiments were also suggested which dealt with the possible effects of calcium in antagonizing the H-ion and with the experimental alteration of physiological condition of animals prior to section.

## MATERIAL AND METHODS

The material used in this investigation was the common flatworm, *Euplanaria dorotocephala,* which is found in great numbers in springs of the Valparaiso moraine to the south and west of Chicago. The chief localities for the collection of worms during this work were Cary, Illinois; Rockford, Illinois; and Valparaiso, Indiana. The worms were kept in large granite pans in the laboratory for several weeks before the experiments were performed. They were fed twice a week on beef liver, and the water was changed on alternate days.

Except when it was desired to bring out the effects of recent feeding, the worms were starved for approximately 2 weeks before they were used in the experiments. (Animals which have been recently fed usually show greater variability in head frequency, as well as a greater susceptibility.) Although 12–14, 14–16, and 15–17 mm. animals were used, by far the most experiments were performed with animals 14–16 mm. in length. Unless particular attention is called to this fact, it may be assumed that 14–16 mm. animals are being used.

Lots of 65 animals of the same length and breadth were chosen from the same stock for tests and controls. The test animals were al-

ways washed twice in the test solution before sectioning. Test animals were sectioned in their respective test solutions, while control animals were sectioned in well water.

In the head-frequency experiments the animals were cut in transverse sections in the manner shown in Figure 1. All the pieces were approximately one-eighth the length of the animal, exclusive of the head. Only the anterior zooids were used, and after a little practice it was possible to cut the pieces with a great deal of exactness. Of the 65 pieces from each region, 15 showing the greatest variations in length were discarded. This procedure considerably decreased any error related to faulty cutting.

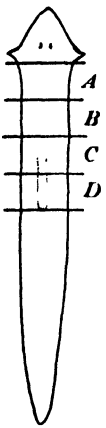


FIG. 1.—Levels *A*, *B*, *C*, and *D* used in all head-frequency experiments. Each section is approximately 1/8th the length of the animal, exclusive of the head. Head and region posterior to the mouth were discarded.

After section the pieces from the different levels were placed in 1-liter Erlenmeyer flasks which were filled with the test and control solutions. The flasks were tightly stoppered and left in the dark at room temperature until the pieces reconstituted. The test and control solutions were changed daily in a great many of the experiments, but in the majority of cases it seemed advisable not to stimulate the pieces by changing the solution. Other reasons for not changing the solutions in which the worms are reconstituting will become clear as the individual experiments are described.

After 2 weeks the reconstituted animals were washed from the flasks and examined under a binocular microscope. They were given arbitrary values according to the type of head as follows: normal, 100; teratophthalmic, 80; teratomorphic, 60; anophthalmic, 40; acephalic, 20. These arbitrary values were multiplied by the frequency of the different head-forms, and the sum of all was divided by the total number of *living* animals to obtain the "head-frequency index." (This "head-frequency index" is identical with the "mean" described by Child and Watanabe, 1935.)

The test solutions were made up in 16-liter quantities as follows:

1. *Carbon dioxide solutions.*—Since the well water in the laboratory contains a large quantity of bicarbonates, it was decided to make use of the $CO_2$ that was liberated when HCl was added to the water. It was found that enough acid added to the well water to change the pH from 7.5 to 5.4 released approximately 60 cc. of $CO_2$ per liter. Changes in pH from 7.5 to 6.0, 7.5 to 6.6, and 7.5 to 7.0 released 45 cc., 18 cc., and 8 cc. of $CO_2$ per liter, respectively. The $CO_2$ liberated is, of course, not all in the gaseous form but is largely in such forms as $H_2CO_3$, $^-HCO_3$, and $^=CO_3$. If the solution was allowed to stand in a container open to the air for a few hours, the $CO_2$ was expelled and the pH of the solution rose to 7.0 or above. As this method for obtaining $CO_2$ in solution was used, all tests involving the use of this reagent are recorded in terms of their pH values.

2. *Hydrogen ion solutions.*—It was highly desirable that the well water in the laboratory be used in making up the test solutions, as it contains the proper salts and was used for the controls. In order to be rid of the effect of $CO_2$ when testing the effect of pH, it was necessary to first free the water of bicarbonates. Smith and Clowes (1924) found that the bicarbonate could adequately be removed from sea water by adding enough concentrated HCl to break down all carbonates and bicarbonates and then removing the $CO_2$ by pumping air through the solution. Hyman (1925) used this method for removing the bicarbonates from well water.

The method used in these experiments for the removal of bicarbonates was similar to Hyman's method. Four and one-half cubic centimeters of concentrated HCl were added to 16 liters of well water, and air was bubbled through this solution for 24 hours. At the end of this time the $CO_2$ in the water was in equilibrium with the air and was found to be of no importance. The buffering capacity of this carbonate-free water almost entirely disappeared. The pH was adjusted to the desired values by adding minute quantities of M/10 HCl or M/10 NaOH. The exact pH value was determined by a quinhydrone apparatus supplemented with a series of La Motte color indicators.

### THE EFFECT OF CARBON DIOXIDE ON HEAD FREQUENCY AND VIABILITY

A number of experiments were performed in determining the exact concentrations of $CO_2$ the animals could tolerate. This procedure was necessary since a large number of experiments have shown that those concentrations which are most effective in altering head frequency are usually very near the lethal level. In fact, it has been found that solutions which cause cytolysis of a small percentage of the experimental pieces are usually the most effective.

Experiments in which intact animals 14–16 mm. in length were placed in $CO_2$ solutions ranging from 4.8 to 7.0 showed that cytolysis in the head regions often took place in concentrations below pH 5.2. Some worms even underwent complete disintegration at these concentrations. One-eighth pieces were quite susceptible to pH 5.2 and usually cytolyzed within a few hours. A concentration in which only a few pieces died was found at pH 5.4–5.6.

It was noted with much interest that animals which had lost only their heads in concentrations of pH 5.2 or below would reconstitute new normal heads in exactly the same solutions that had caused the cytolysis of the old head. It was also noted that those animals which had lost their heads, as well as animals whose heads had remained intact, often underwent fission in high concentrations of $CO_2$. The percentage of fission was apparently closely related to the size and general physiological condition of the worms.

Another phenomenon, which was noted at this time and later made use of in experiments on "conditioning," was the acquiring of tolerance by intact animals to $CO_2$. It was found that animals which had been kept in solutions of pH 5.4–5.6 for a few days could live, with no apparent injury, when transferred to solutions of pH 5.2. If kept in the latter solution for a few days, they could then be transferred to pH 5.0 without cytolysis resulting. In other words, animals living in certain concentrations of $CO_2$ for a time will acquire a tolerance for this reagent and, in doing so, will be able to survive solutions that are toxic to animals taken directly from well water, pH 7.5.

In the experiments on head frequency described in this section only worms which had been kept in freshly drawn well water until the time for experimentation were used. In the early experiments the solutions were changed daily; but, because of the large quantity of solution, as compared to the pieces, and the fact that there was little or no change in the solutions during the reconstitutional period, this was given up as time-consuming. Other reasons for not changing the solutions were that the type of head appears to be determined by at least 2 or 3 days after section and the stimulation of pieces in

TABLE I

HEAD FREQUENCY AND VIABILITY OF *Euplanaria dorotocephala* IN CARBON DIOXIDE SOLUTIONS

(Head types and deaths given in percentages)

| | Well Water (Control) | | | | Carbon Dioxide Solution, pH 5.4–5.6 | | | |
|---|---|---|---|---|---|---|---|---|
| | *A* | *B* | *C* | *D* | *A* | *B* | *C* | *D* |
| Normal | 92.0 | 34.6 | 7.3 | 2.6 | 72.0 | 60.0 | 18.6 | 13.3 |
| Teratophthalmic | 8.0 | 40.0 | 5.3 | 0.6 | 5.3 | 14.0 | 20.0 | 8.0 |
| Teratomorphic | ...... | 2.0 | 4.6 | ...... | 1.3 | 0.6 | 2.6 | 4.6 |
| Anophthalmic | ...... | 10.0 | 21.3 | 10.3 | ...... | 4.6 | 15.3 | 6.0 |
| Acephalic | ...... | 10.6 | 60.6 | 86.6 | 1.3 | 10.0 | 29.3 | 22.6 |
| Dead | ...... | 2.0 | 0.6 | ...... | 20.0 | 10.0 | 14.6 | 45.3 |
| Index | 98.4 | 75.9 | 35.3 | 23.9 | 96.6 | 84.6 | 55.8 | 53.6 |

changing the solutions might present additional, not completely controllable factors.

In Table I are presented the head frequencies of three different experiments involving 150 test and 150 control worms. These experiments were performed on three different stocks and at three different times; but as each experiment shows the same relationship between tests and controls, these three different experiments are grouped together. It can readily be seen from this table that the head frequencies are distinctly higher in *B*, *C*, and *D* pieces which have reconstituted in the test solution. The relationship between tests and controls is shown graphically in Figure 2, in which the head frequency indices are plotted against body-level.

Another fact brought out in Table I is the different percentages of viability of pieces from different levels. The data at least indicate that *A* and *D* pieces are more susceptible to the toxic effect of $CO_2$ than are pieces *B* and *C*. It may be that *A* pieces are more susceptible because of the normal higher activity of that particular level. The high susceptibility of *D* pieces may be attributed to the tremendous increase in respiratory metabolism and in susceptibility which these levels undergo following section. The presence of the pharynx in *D* pieces, may also have something to do with the greater susceptibility.

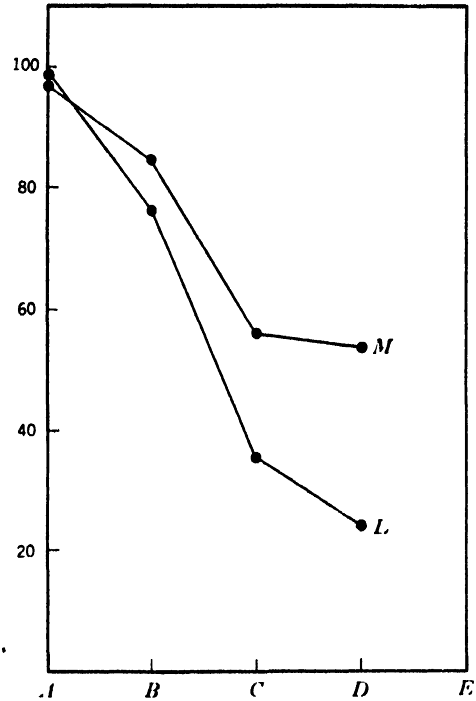


FIG. 2.—Head frequencies in $CO_2$ solution (*M*) and well water (*L*). Graph is plotted with the head-frequency indices as ordinates and body-levels as abscissae.

Experiments on head frequency were performed in which the concentrations of $CO_2$ were pH 5.4, 6.2, and 6.6. The head-frequency indices and the percentage of viability are shown in Table II. These worms were of the same size and from the same stock. They were sectioned at the same time and serve as adequate controls for each other.

It will be noted that pieces which have reconstituted in pH6.2 show a head frequency as high as, or possibly higher than, those which have reconstituted in pH 5.4; yet the number of deaths is practically zero. Solutions with a pH of 5.4 contain approximately 60 cc. of $CO_2$ per liter, while those with a pH of 6.2 contain approxi-

mately 38 cc. per liter. It appears from these data that pH values below 6.2 are more effective in increasing the number of deaths than the head frequency. At pH 6.6 the effect of $CO_2$ is still evident at *C* and *D* levels.

That this effect we have been describing is actually due to $CO_2$ rather than the H-ion is shown in Table III, in which the head-frequency indices of worms which have reconstituted in carbonate-free well water, pH 5.6 and 7.5, and in unaltered well water (control solutions), pH 7.5, are compared. According to Table III, the lots in carbonate-free well water at pH 7.5 and 5.6 show slightly lower head frequencies than the control, but the significance of the differences is doubtful. That the effect of H-ion alone at these concentrations is unimportant is fully confirmed by later experiments.

TABLE II

HEAD FREQUENCY AND VIABILITY OF *Euplanaria dorotocephala* IN VARIOUS CONCENTRATIONS OF CARBON DIOXIDE

| | HEAD-FREQUENCY INDEX | | | | PERCENTAGE LIVING | | | |
|---|---|---|---|---|---|---|---|---|
| | *A* | *B* | *C* | *D* | *A* | *B* | *C* | *D* |
| Well water (control) | 97.4 | 82.4 | 39.2 | 27.2 | 100.0 | 98.0 | 100.0 | 100.0 |
| $CO_2$ solution, pH 5.4 | 91.5 | 89.2 | 64.4 | 58.7 | 80.0 | 82.0 | 72.0 | 62.0 |
| $CO_2$ solution, pH 6.2 | 99.6 | 96.4 | 77.6 | 51.0 | 100.0 | 100.0 | 100.0 | 98.0 |
| $CO_2$ solution, pH 6.6 | 97.4 | 81.2 | 47.0 | 42.0 | 100.0 | 100.0 | 100.0 | 100.0 |

TABLE III

HEAD FREQUENCY AND VIABILITY OF *Euplanaria dorotocephala* IN CERTAIN NON-EFFECTIVE HYDROGEN ION SOLUTIONS

| | HEAD-FREQUENCY INDEX | | | | PERCENTAGE LIVING | | | |
|---|---|---|---|---|---|---|---|---|
| | *A* | *B* | *C* | *D* | *A* | *B* | *C* | *D* |
| Well water (control) | 98.8 | 81.6 | 41.0 | 31.8 | 100.0 | 100.0 | 72.0 | 100.0 |
| Carbonate-free well water, pH 7.5 | 98.8 | 68.0 | 36.0 | 30.8 | 100.0 | 100.0 | 100.0 | 100.0 |
| Carbonate-free well water, pH 5.6 | 99.0 | 78.0 | 34.6 | 27.4 | 100.0 | 100.0 | 100.0 | 100.0 |

### THE EFFECT OF THE HYDROGEN ION ON HEAD FREQUENCY AND VIABILITY

All test animals dealt with in this section were allowed to reconstitute in carbonate-free well water which was prepared by the method described earlier in this paper.

Experiments on intact animals submitted to different concentrations of the H-ion show that this reagent has little effect between pH values of 7.2 and 4.2. Below 4.1 all of the animals usually cytolyzed. There was no evidence at this time of any acclimation to high concentrations, and no increase in fission rate was noted. If animals are left in pH 4.15 for several days, the pigmentation darkens and the tissues appear somewhat swollen. In alkaline solutions the animals died at a pH of approximately 10.0. In these solutions there was no evidence of any physiological changes before death.

A series of fifteen experiments was devised in which the head frequencies of the different levels of 50 animals were determined at every 0.4 pH value between pH 4.2 and 9.6. The solutions were changed daily throughout the entire reconstitutional period. The worms used in these experiments were from the same lot and were sectioned at approximately the same time. From these fifteen experiments the only concentration that gave any evidence of inducing an increase in head frequency was pH 4.2. There was no effect whatever between pH 4.6 and 7.0. From 7.0 to 9.6 there appeared to be a slight decrease in the head frequencies of reconstituted *C* pieces.

As a pH of approximately 4.2 seemed to be quite important in the alteration of physiological conditions in reconstituting pieces, twelve different experiments, involving over 1,200 worms, were performed at pH ranges from 4.15 to 4.30. Each experiment consisted of 50 test and 50 control worms. These animals were from different localities and were collected at different times throughout the year. Although the head frequencies of the animals from different stocks did not always coincide, the head frequencies of the tests were always well above the head frequencies of the controls. The data from these different experiments were therefore grouped together in Table IV.

It will be noted from Table IV that the percentage of normal worms is much greater in the tests than in the controls, except in *A* pieces, and that the percentage of acephalic forms in the tests is

much smaller. A comparison of the head-frequency indices of worms which have reconstituted in critical concentrations of $CO_2$ (Table I) and of H-ion (Table IV) indicates that in these concentrations the H-ion may be more effective in increasing head frequency than $CO_2$. It will also be noted that the death frequency in the tests at the different levels corresponds to the death frequency of pieces in high concentrations of $CO_2$.

TABLE IV

HEAD FREQUENCY AND VIABILITY OF *Euplanaria dorotocephala* IN CARBONATE-FREE WELL WATER

(Head types and deaths given in percentages)

| | WELL WATER (CONTROL) | | | | CARBONATE-FREE WELL WATER, pH 4.15-4.3 | | | |
|---|---|---|---|---|---|---|---|---|
| | *A* | *B* | *C* | *D* | *A* | *B* | *C* | *D* |
| Normal | 91.1 | 43.7 | 13.0 | 8.3 | 76.6 | 51.2 | 35.8 | 35.7 |
| Teratophthalmic | 7.7 | 27.7 | 11.3 | 7.5 | 6.2 | 22.7 | 19.2 | 14.3 |
| Teratomorphic | 1.0 | 2.3 | 4.2 | 3.5 | 0.2 | 2.7 | 3.8 | 6.3 |
| Anophthalmic | 0.2 | 9.6 | 20.2 | 9.0 | 0.2 | 4.1 | 13.8 | 9.3 |
| Acephalic | ...... | 16.6 | 50.8 | 71.2 | ...... | 7.5 | 16.8 | 13.2 |
| Dead | ...... | ...... | 0.5 | 0.5 | 16.6 | 11.8 | 10.5 | 21.2 |
| Index | 97.9 | 74.4 | 43.0 | 34.3 | 98.1 | 84.0 | 69.6 | 72.7 |

Pieces that have reconstituted under conditions of high H-ion concentrations (pH 4.15) show morphological characteristics which are quite different from the controls. The pigmentation in the old tissues of the reconstituted test worms usually becomes very dark and diffuse. The new outgrowth may remain almost entirely unpigmented for a considerable length of time. Stunting of new tissue in both head and tail regions has been noted in such high concentrations of the H-ion.

## CALCIUM ANTAGONISM TO THE HYDROGEN ION

Much work has been done in the past on the antagonism of ions. It has been found, in general, that the injurious effect of one ion may be obliterated by another ion even though the other ion alone may produce injuries. Divalent ions have been found to antagonize the actions of monovalent ions to a great extent. Calcium, particularly,

has been used in many fields of biological investigation to antagonize the injurious effects of sodium and potassium.

It has been established, in this work, that the H-ion in high concentrations has the property of altering reconstitutional development in *Euplanaria*. The question arises as to whether or not the effect of this ion can be antagonized by increasing the calcium content of the test solutions.

Preliminary experiments were carried out on intact worms in which from 0.1 to 1.0 gm. of calcium ($CaCl_2$, anhydrous) were added per liter of test solution. The pH of the solutions was approximately 4.0. It was found that animals in solutions of the same pH would live a much longer time if the calcium concentration was increased. It apparently made little difference whether the calcium concentration was increased 0.1 or 1.0 gm. per liter.

A series of experiments was next conducted to determine if calcium could antagonize the effect of H-ion on head frequency. Six experiments were performed, which involved 900 worms from the same stock. Each experiment consisted of a control in well water, pH 7.5, a control in carbonate-free well water, pH 4.15, and a test in carbonate-free well water, pH 4.15, plus 0.5 gm. of $CaCl_2$ per liter. The results of these six experiments are tabulated in Table V. It will be noted from this table that worms allowed to reconstitute in carbonate-free well water at pH 4.15 have a characteristically higher head frequency than those which have reconstituted in well water, pH 7.5. This is not true in the case of pH 4.15, where the calcium concentration of the solution has been increased. Instead, the head frequency in these solutions approaches very closely the head frequency obtained in the well-water controls. In other words, the addition of calcium to these concentrations of the H-ion almost completely antagonized its effect on head frequency. Not only is this effect of the H-ion on head frequency decreased with calcium, but the death percentages of the pieces are likewise decreased under the same conditions.

In the light of the foregoing observations, a head-frequency experiment was performed, using as control solutions well water, pH 7.5, and carbonate-free well water, pH 4.1. The test solution was carbonate-free well water, pH 4.1, plus 0.5 gm. of $CaCl_2$ per liter.

The head frequencies and viability in this experiment are recorded in Table VI. It will be seen, in this case, that calcium antagonizes to a high degree the death-rate in high concentrations of H-ion but that the increase in head frequency is only partially antagonized.

TABLE V

ANTAGONIZING ACTION OF CALCIUM ON EFFECTIVE CONCENTRATIONS OF THE HYDROGEN ION

(Head types and deaths given in percentages)

| | Well Water (Control) | | | | Carbonate Free Well Water, pH 4.15 | | | | Carbonate-Free Well Water, pH 4.15, Plus 0.5 Gm. $CaCl_2$ (*L*) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *A* | *B* | *C* | *D* | *A* | *B* | *C* | *D* | *A* | *B* | *C* | *D* |
| Normal | 92 | 21 | 3 | 4 | 78 | 40 | 21 | 23 | 93 | 36 | 4 | 4 |
| Teratophthalmic | 7 | 28 | 3 | 1 | 4 | 28 | 16 | 8 | 5 | 30 | 4 | 2 |
| Teratomorphic | 1 | 3 | 1 | 1 | .. | 3 | 6 | 6 | .... | 5 | 4 | 1 |
| Anophthalmic | .... | 16 | 12 | 4 | ... | 7 | 16 | 12 | .... | 11 | 23 | 7 |
| Acephalic | .... | 31 | 80 | 88 | .. | 14 | 32 | 20 | ... | 20 | 65 | 83 |
| Dead | ... | 1 | ... | 2 | 18 | 9 | 10 | 31 | 2 | .... | .... | 2 |
| Index | 98 | 59 | 27 | 25 | 99 | 76 | 55 | 61 | 99 | 70 | 32 | 27 |

TABLE VI

THE PREVENTION OF DEATH BY THE HYDROGEN ION WITH CALCIUM

| | Head-Frequency Index | | | | Percentage Living | | | |
|---|---|---|---|---|---|---|---|---|
| | *A* | *B* | *C* | *D* | *A* | *B* | *C* | *D* |
| Well water (control) | 97.9 | 44.5 | 21.6 | 20.8 | 98.0 | 98.0 | 100.0 | 100.0 |
| Carbonate-free well water, pH 4.1 | ...... | ...... | 90.0 | ...... | 0.0 | 0.0 | 8.0 | 0.0 |
| Carbonate-free well water, pH 4.1, plus 0.5 gm. $CaCl_2$ (*L*) | 99.0 | 81.7 | 40.0 | 42.9 | 74.0 | 94.0 | 92.0 | 96.0 |

It was of interest in considering the subject of ionic antagonism to test the viability and head frequency of highly susceptible animals (those fed 48–72 hours prior to section) in concentrations of H-ion, pH 4.15, with and without additional calcium. The results are shown in Table VII. In this case the death-rate is considerably antagonized,

while the head frequency is only partially antagonized by the action of calcium.

In order to test the effect of increased concentrations of calcium, aside from the antagonistic action on the H-ion, a series of experi-

TABLE VII

THE EFFECTS OF CALCIUM AND THE HYDROGEN ION ON HIGHLY SUSCEPTIBLE ANIMALS

| | Head-Frequency Index | | | | Percentage Living | | | |
|---|---|---|---|---|---|---|---|---|
| | *A* | *B* | *C* | *D* | *A* | *B* | *C* | *D* |
| Well water (control) ..... | 100.0 | 65.3 | 34.1 | 28.3 | 90 0 | 84.0 | 88 0 | 96.0 |
| Carbonate-free well water, pH 4.15 .............. | 100 0 | 80 0 | 56 0 | 46.6 | 8.0 | 4.0 | 10.0 | 12 0. |
| Carbonate-free well water, pH 4.15, plus 0.5 gm. $CaCl_2$ (*L*) ............. | 99.2 | 64 0 | 45 3 | 41 3 | 100 0 | 90 0 | 98.0 | 90.0 |

TABLE VIII

THE ACTION OF CALCIUM ALONE ON HEAD FREQUENCY AND VIABILITY IN *Euplanaria dorotocephala*

| | Head-Frequency Index | | | | Percentage Living | | | |
|---|---|---|---|---|---|---|---|---|
| | *A* | *B* | *C* | *D* | *A* | *B* | *C* | *D* |
| Well water, pH 7.5 (control) ..... .......... | 99.2 | 64.4 | 22.5 | 20 0 | 100 0 | 100.0 | 96.0 | 96.0 |
| Well water, pH 7.5, plus 0.5 gm. $CaCl_2$ (*L*) ...... | 100.0 | 71 2 | 24 7 | 21 2 | 100 0 | 100.0 | 100.0 | 100.0 |
| Carbonate-free well water, pH 4.15 .............. | 100.0 | 87.6 | 64.5 | 51 2 | 98.0 | 100.0 | 98.0 | 68 0 |
| Carbonate-free well water, pH 4.15, plus 0.5 gm. $CaCl_2$ (*L*) ............. | 99.6 | 77 2 | 32.2 | 26 4 | 100 0 | 100.0 | 98.0 | 100.0 |

ments was performed in which the test solution was well water, pH 7.5, plus 0.5 gm. of $CaCl_2$ per liter. The control solutions were well water, pH 7.5, carbonate-free well water, pH 4.15, and carbonate-free well water, pH 4.15, plus 0.5 gm. of $CaCl_2$ per liter. The results, which are tabulated in Table VIII, indicate that calcium alone may slightly increase the head frequency. In this case it apparently has little effect on viability.

One head-frequency experiment was performed in which $CaCl_2$ (0.5 gm. per liter) was added to a test solution of $CO_2$ pH 5.4. This experiment was adequately controlled with solutions of $CO_2$, pH 5.4, and well water, pH 7.5. It was found that calcium did not antagonize the effects of $CO_2$ either by decreasing the head frequency or the death percentages.

### THE PHYSIOLOGICAL CONDITION OF *Euplanaria* IN NATURE

Throughout this investigation it has been found that control worms of the same size but from different stocks may yield very different head frequencies.

It has also been noted that worms from the same stock vary considerably from time to time as regards head frequency. Animals collected in the summer may show a different head frequency than those collected in the winter. It has been observed that worms in "poor" condition (i.e., many cytolyzing in the stock pans) usually show a higher head frequency than worms from the same stock under better conditions.

Worms of the same size and kept under the same laboratory conditions may behave differently if they are originally from different localities. In Figure 3 are plotted the head-frequency indices of 150 control worms from each of three widely separated places.

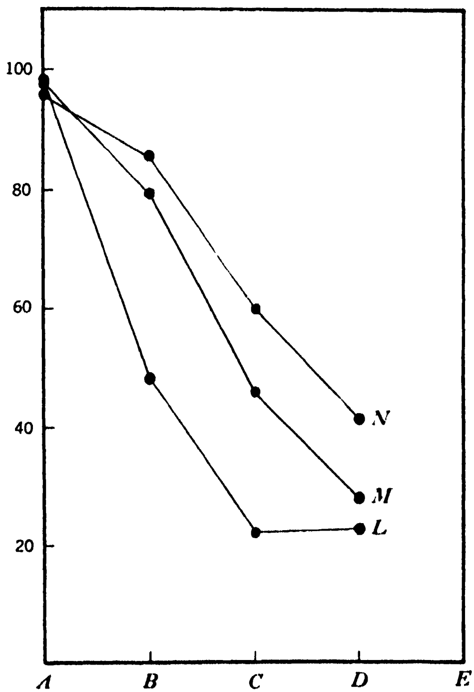


FIG. 3.—Head frequencies of different stocks of *Euplanaria dorotocephala* which have reconstituted in well water. Stocks were from Valparaiso, Indiana (*L*); Cary, Illinois (*M*); and Rockford, Illinois (*N*.)

It is not intended to imply by this graph that worms from different localities are persistently different. The head frequencies of worms (from any one locality) at different times in the year may vary as much as the three different head frequencies shown in the graph. The only object of the graph is to show that the different lots of worms used in our experiments may be in different physiological conditions and that these conditions are expressed through the head frequencies. This difference in physiological condition must have been impressed upon the worms by their different environments prior to section.

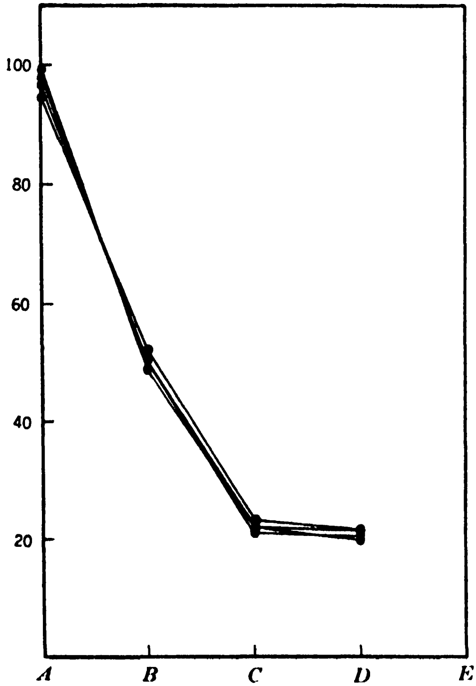


FIG. 4.—Head frequencies of four lots of worms from the same stock. They were identical in size and condition and were sectioned at approximately the same time. All were allowed to reconstitute in well water at the same temperature. (Worms were from Valparaiso, Indiana.)

But is there any pronounced difference in head frequency of different lots of worms of the same size, from the same stock, collected at the same time, sectioned at approximately the same time and kept under the same laboratory conditions? The answer to this question is given in Figure 4, in which the head-frequency indices of four control lots of 50 worms per lot, which met all the requirements, are plotted. It must be concluded from these data that like worms give like head frequencies and that all experiments on the alteration of head frequency have been adequately controlled.

It has been repeatedly observed that worms with controls of relatively high head frequency respond to the effects of such reagents as

H-ion and $CO_2$ with a greater increase in head frequency than stocks in which the controls show a low head frequency. Apparently animals in good condition (with low head frequency), although more susceptible than those in poor condition to directly lethal agents, are more capable of maintaining approximately normal internal conditions or of regulating these conditions when subjected to $CO_2$ or H-ion in the non-lethal concentrations used in these experiments. In other words, the animals in good condition tolerate these concentrations better, and so appear to possess a greater physiological stability within the experimental limits than those in poor condition.

## THE EXPERIMENTAL ALTERATION OF PHYSIOLOGICAL CONDITION IN *Euplanaria*

Since worms of the same size but from different environments yield different head frequencies under the same experimental conditions, a number of experiments were performed to determine whether the physiological conditions of animals could be altered in the laboratory in a short time by experimental means.

Three different methods for altering the physiological condition of the animals were tested. These were as follows:

1. Lots of 500–700 worms (14–16 mm.) were placed in a tightly stoppered 18-liter bottle in a solution of $CO_2$, pH 5.6–5.7, for 4 days. This was followed by 6 days in $CO_2$ solution, pH 5.05–5.2. At the end of the conditioning period the worms were used for experimentation.

2. Lots of 500–700 worms (14–16 mm.) from the same stock as the worms in (1) were placed in carbonate-free well water, pH 4.15, in a tightly stoppered 18-liter bottle. The worms were left in this solution 9–10 days before sectioning for head frequency. The solution was changed daily.

3. Lots of worms of the same size and from the same stock as (1) and (2) were placed in well water, pH 7.5, in a tightly stoppered 18-liter bottle. The worms were kept under these conditions for 10 days prior to sectioning. The water was not changed from the beginning to the end of the conditioning period.

A control lot of 500–700 worms of the same size and from the same stock was maintained in an open pan, and the water changed daily.

Animals treated in the foregoing manner were taken directly from the experimental solutions, sectioned, and allowed to reconstitute in well water, pH 7.5, in carbonate-free well water, pH 4.15, and in carbon-free well water, pH 4.15, plus 0.5 gm. of $CaCl_2$ per liter. The head frequencies were compared with non-treated control worms which had been submitted to the same test solutions during re-constitution.

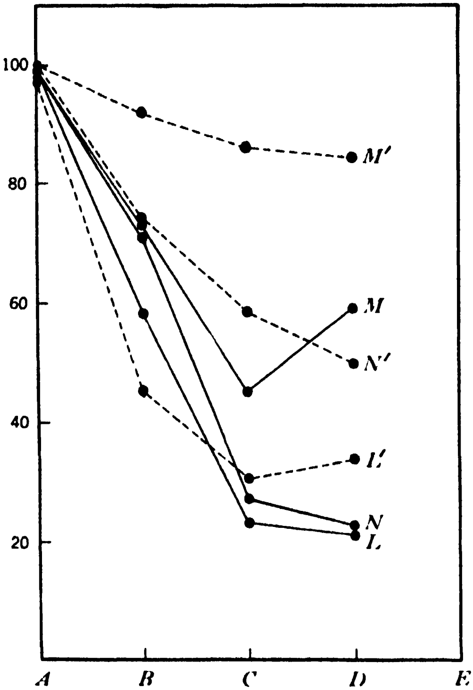


FIG. 5.—Head frequencies of $CO_2$ conditioned worms (broken lines) under different experimental and control conditions, as compared to non-conditioned worms. *L*, non-conditioned worms which have reconstituted in well water. *L'*, conditioned worms which have reconstituted in well water. *M*, non-conditioned worms which have reconstituted in carbonate-free well water, pH 4.15. *M'*, conditioned worms which have reconstituted in carbonate-free well water, pH 4.15. *N*, non-conditioned worms which have reconstituted in carbonate-free well water, pH 4.15, plus 0.5 gm. of $CaCl_2$ per liter. *N'*, conditioned worms which have reconstituted in carbonate-free well water, pH 4.15, plus 0.5 gm. of $CaCl_2$ per liter.

1. *The effect of carbon dioxide on physiological condition.*—Animals conditioned with $CO_2$ gave by far the best results and showed clearly how the physiological gradients may be altered in intact animals before section.

*Euplanaria* conditioned to $CO_2$ and allowed to reconstitute in well water shows a higher head frequency in *C* and *D* pieces. In this experiment the conditioned *B* pieces may show a lower head frequency than the controls, as shown in Figure 5 (*L*, *L'*).

Carbon dioxide conditioned worms which had reconstituted in carbonate-free well water, pH 4.15, showed a considerably higher

head frequency than non-conditioned worms in the same solution (Fig. 5 *M*, *M′*). In the non-conditioned worms the percentages of deaths were greater in *A* and *D* pieces. In the conditioned worms the death percentages (although as high) were not distributed in a regular fashion.

The non-conditioned worms reconstituting in pH 4.15 plus 0.5 gm. of $CaCl_2$ per liter (Fig. 5 *N*) give a head frequency almost as low as the non-conditioned worms reconstituting in well water (Fig. 5 *L*). In the conditioned worms (Fig. 5 *N′*), however, the antagonism of calcium to the H-ion decreases the head frequency below that of conditioned worms in H-ion alone (Fig. 5 *M′*), but it remains far above that of the non-conditioned worms (Fig. 5 *N*). Evidently the antagonism of calcium to the H-ion is not complete as regards head frequency in animals conditioned to $CO_2$.

An interesting series of head-frequency experiments was performed on a separate lot of $CO_2$ conditioned worms in which pieces from the different levels were placed in $CO_2$ solution, pH 5.35, for given intervals of time after section. The pieces were then returned to well water, pH 7.5. In this series the reconstitution of the conditioned worms took place in (1) well water, pH 7.5, for the entire period, (2) $CO_2$ solution for the first 24 hours, (3) $CO_2$ solution for the first 48 hours, (4) $CO_2$ solution for the first 72 hours, and (5) $CO_2$ solution for the entire period. The head-frequency indices are presented in Table IX.

It will be noted from these data that all levels which have undergone $CO_2$ treatment, either before or after section, show an increased head frequency. It is of particular interest that conditioned pieces show a much higher head frequency when submitted to $CO_2$ for only 24–48 hours after section than with longer periods. (This effect may be because of a differential recovery or an activation of the head-forming cells that has been brought about by the change from an inhibiting solution to well water. If this is the case, we may assume that the exposure time was sufficient to inhibit the posterior-cut-surface factor.)

Animals conditioned in $CO_2$, sectioned, and allowed to reconstitute for the entire period in $CO_2$ solution show a lower head frequency than would be expected from the results with non-condi-

tioned worms. This fact suggests that an acquired tolerance to $CO_2$ before section may result in less effect of $CO_2$ after section.

2. *The effect of the hydrogen ion on physiological condition.*—Animals conditioned with the H-ion and those not conditioned gave almost identical head frequencies when allowed to reconstitute in well water, pH 7.5. When reconstitution took place in carbonate-free well water, pH 4.15, the conditioned animals showed a distinctly higher head frequency in *B*, *C*, and *D* pieces than non-conditioned animals in the same solution.

TABLE IX

HEAD FREQUENCY AND VIABILITY OF ANIMALS CONDITIONED TO CARBON DIOXIDE AND ALLOWED TO RECONSTITUTE FOR VARIOUS INTERVALS OF TIME IN THE SAME SOLUTION

| | Head-Frequency Index | | | | Percentage Living | | | |
|---|---|---|---|---|---|---|---|---|
| | *A* | *B* | *C* | *D* | *A* | *B* | *C* | *D* |
| Well water (control) | 97.9 | 44.5 | 21.6 | 20 8 | 98.0 | 98.0 | 100 0 | 100.0 |
| T-1, well water, pH 7.5 | 100.0 | 58.8 | 41 2 | 30 4 | 100 0 | 100 0 | 100.0 | 100.0 |
| T-2, $CO_2$ solution, pH 5.35, 24 hours only | 100.0 | 88.6 | 70 4 | 70 8 | 90.0 | 88.0 | 92.0 | 74.0 |
| T-3, $CO_2$ solution, pH 5.35, 48 hours only | 100.0 | 90.9 | 73 2 | 79 4 | 96.0 | 100 0 | 82.0 | 68.0 |
| T-4, $CO_2$ solution, pH 5.35, 72 hours only | 100.0 | 86.5 | 65.1 | 59 6 | 100 0 | 98.0 | 94.0 | 92.0 |
| T-5, $CO_2$ solution, pH 5.35, entire period | 99.6 | 84.9 | 65 8 | 58 8 | 98.0 | 82.0 | 90.0 | 90.0 |

Worms conditioned with the H-ion and allowed to reconstitute in pH 4.15 plus 0.5 gm. of $CaCl_2$ per liter gave a very interesting result. Instead of calcium causing a decrease in head frequency, as it did in the case of non-conditioned worms, it actually caused an *increase* in head frequency at all four levels. In other words, in the case of H-ion conditioned worms, the head frequency in high concentrations of the H-ion with calcium was higher than in the same concentrations of H-ion without calcium.

Death of pieces in pH 4.15 was almost entirely prevented by conditioning the worms to H-ion prior to section.[3]

[3] Previous experiments with whole animals had given no visible evidence of an acquirement of tolerance to high concentrations of the H-ion. These experiments indicate,

3. *The effect of standing well water on physiological condition.*—Experiments comparable to those performed on worms conditioned to the H-ion and $CO_2$ were performed on worms conditioned to standing well water. It may be briefly stated that the head frequencies of worms conditioned in this manner were not substantially different from those of non-conditioned worms.

### THE EFFECT OF THE HYDROGEN ION ON DIFFERENTIALS IN RATE OF EYESPOT FORMATION

It has been shown quite definitely throughout this investigation that there actually is a head-frequency gradient in *Euplanaria* and that this gradient can be altered by means of different reagents in the external medium. In this section we are not concerned with the head frequency except to point out the parallelism between it and another gradient, namely, the gradient in rate of eyespot formation. In speaking of the different gradients it is not meant to imply that there are different physiological gradients but that the different phenomena observed are different manifestations of the same axial differential.

Watanabe (1935*a*) has shown a gradient in rate of eyespot formation in this species when the different levels were allowed to reconstitute in well water. The object of the investigation here described is to determine whether external reagents can alter the eyespot gradient in a manner analogous to the alteration of the head-frequency gradient.

Animals in lots of 50 were sectioned in the manner shown in Figure 6. The section designated *X* comprises the anterior 5/16ths of the animal (exclusive of the head), while *Y* is the 5/16ths of the animal immediately posterior to *X*. Controls reconstituted in well water, pH 7.5, while the tests were submitted to carbonate-free well water, pH 4.20–4.15, for the reconstitutional period. The pieces were first examined at 36 hours after section. They were then examined every 12 hours until all showed definite eyespots. All examinations were made with the aid of a compound microscope. All the pieces of the size here described reconstituted into animals with normal heads.

however, that there actually is some readjustment of the intact animals to pH. It appears from these data that the acquirement of tolerance for the H-ion is considerably less than for $CO_2$.

It is sometimes quite difficult, especially in *A* pieces, to tell with certainty the exact time of the first appearance of the eyespots. This is primarily due to the accumulation of pigment at the site of injury, which renders the minute eyespot invisible. Pieces recorded as having eyes at any particular time always showed the eyespots distinctly.

By recording the number of animals that had developed eyes during each 12-hour interval, it was possible to get a mean rate of eyespot formation with a standard error. From data obtained from the different levels under control and experimental conditions it was possible to demonstrate any differential in rate of eyespot formation, as well as any alterations which may have been caused by external factors.

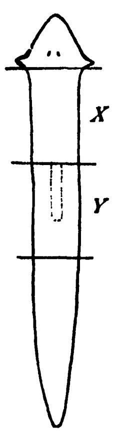


FIG. 6.—Sections *X* and *Y* (approximately 5/16ths of animal, exclusive of head) used in experiments on gradient in rate of eyespot formation.

The data for the rate of eyespot formation in pieces *X* and *Y*, as well as the head frequencies of 1/8th pieces (*A*, *B*, *C*, *D*, Fig. 1), from the same stocks of animals sectioned at the same time and allowed to reconstitute under the same test and control conditions are presented in Table X. In the first section of Table X are given the head-frequency indices. In the second section are given the rates of eyespot appearance in pieces *X* and *Y*. In the third section are given the differences in rate of eyespot appearance between (1) *X* and *Y* levels of both tests and controls ($Y-X$), (2) between *X* levels of tests and *X* levels of controls ($X_{II}-X_{I}$), (3) between *Y* levels of tests and *Y* levels of controls ($Y_{II}-Y_{I}$), and (4) between the differences found in (2) and (3) $[(X_{II}-X_{I})-(Y_{II}-Y_{I})]$.

The first experiment on rate of eyespot formation was performed in midwinter at a temperature of approximately 20° C. It will be noted (Lot 1, Table X) that the control worms showed a relatively high head frequency, while the differential in rate of eyespot formation between levels *X* and *Y* was quite small (3.6±0.95 hours). In the test worms the head-frequency gradient had almost entirely dis-

appeared, and the eyespot gradient was of no significance (0.7 ± 1.43 hours). In comparing the rate of eyespot formation in the tests and controls at the same levels, it was found that the rate of development was definitely inhibited in high concentrations of the H-ion (10 ± 1.39 hours for $X$ levels and 6.3 ± 1.14 hours for $Y$ levels). It appears from these data that the rate of eyespot formation was inhibited to a

TABLE X

THE GRADIENT IN RATE OF EYESPOT FORMATION IN *Euplanaria dorotocephala* WITH ITS ALTERATION BY USE OF THE HYDROGEN ION

(Rate of eyespot appearance given in hours)

| | | Head-Frequency Index | | | | Rates of Eyespot Appearance | | Differences in Rate of Eyespot Appearance | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $A$ | $B$ | $C$ | $D$ | $X$ | $Y$ | $Y-X$ | $X_{II}-X_I$ | $Y_{II}-Y_I$ | $(X_{II}-X_I)-(Y_{II}-Y_I)$ |
| Lot 1 | I. Well water (control) | 99 | 97 | 63 | 51 | 73.9 ±0.82 | 77.5 ±0.49 | 3.6 ±0.95 | 10 6 ±1.39 | 6 3 ±1.14 | 4.3 ±1.71 |
| | II. Carbonate-free well water, pH 4.2 | 98 | 97 | 93 | 91 | 84.5 ±0.98 | 83.8 ±1.03 | −0 7 ±1.43 | | | |
| Lot 2 | I. Well water (control) | 99 | 49 | 21 | 22 | 50.4 ±0.86 | 64.1 ±1.05 | 13.7 ±1.35 | 9 4 ±1.54 | 3 8 ±1.56 | 5.6 ±2 2 |
| | II. Carbonate-free well water, pH 4.15 | 96 | 63 | 34 | 44 | 59 8 ±1.28 | 67.9 ±1.16 | 8.1 ±1.73 | | | |

greater extent in $X$ than in $Y$ pieces (4.3 ± 1.71 hours). It seems to be quite clear that in the test solutions the physiological gradient may be flattened out or obliterated through differential inhibition.

The second experiment on rate of eyespot formation (Lot 2, Table X) was performed in the summer at a temperature of approximately 32° C. It will be noted that the control worms showed a very low head frequency, while the differential in rate of eyespot formation in pieces $X$ and $Y$ of similar worms was quite marked (13.7 ± 1.35 hours). The test worms (those which reconstituted in carbonate-free well water, pH 4.15) showed a higher head frequency in pieces $B$, $C$, and $D$ and less differential in rate of eyespot formation

at levels $X$ and $Y$ ($8.1 \pm 1.73$ hours). As in the case of the foregoing experiment, both levels are retarded in rate of eyespot formation ($9.4 \pm 1.54$ hours for level $X$ and $3.8 \pm 1.56$ hours for level $Y$). Here, again, level $X$ is retarded more than level $Y$ ($5.6 \pm 2.2$ hours). In this later experiment the differential was so great between the different levels that it was only partially destroyed by the H-ion.

## DISCUSSION

It has been pointed out repeatedly during this work that the inhibition of heads in short transverse pieces of *Euplanaria* is the result of some factor or factors arising from the posterior cut surface. This inhibiting influence may be present for several days and, as stated by Child and Watanabe (1935) and Watanabe (1935*b*), it exerts its effect by preventing in some way the dedifferentiation and activation of the head-forming cells in the anterior region of the piece. These authors have given evidence indicating that this inhibitory effect of posterior cut surface is transmitted forward from its point of origin chiefly by way of the ventral nerve cords.

It has also been found (Watanabe, 1935*a*) that a gradient in rate of head growth (eyespot formation) exists along the axis. This gradient of eyespot formation is apparently independent of any posterior effect, since it appears in long pieces in which head development is not inhibited. Those regions which show the slowest rate in eyespot formation are likewise the regions which give the largest number of inhibited head-forms with short transverse pieces.

It has been suggested (Child and Watanabe, 1935) that the inhibitory effect arising from the posterior cut surface may not come into play in transverse pieces from the more anterior levels because of a greater activity of the head-forming cells in these regions. It was also suggested that a decrement in the transmission of the inhibiting factor may be greater in the anterior levels and less in the posterior levels of the first zooid.

When anterior and posterior section are made at the same time in 1/8th $D$ pieces, head frequency is low and acephalic forms predominate; while a delay in the posterior cut for 6–8 hours will result in the reconstitution of animals with 100 per cent heads, and with longer delay 100 per cent normal heads. From this it seems to be evident

that a short delay in bringing into action by posterior section the factor inhibiting head development is enough to permit at least a beginning in dedifferentiation and activation of the head-forming cells. With this advantage the anterior end is able to carry through the reconstitution of a normal head in spite of any inhibiting effect which later may be imposed from the posterior cut surface.

It seems quite clear that the increase in head frequency found in the experiments with $CO_2$ and H-ion is brought about through some kind of inhibition of the factor inhibiting head development. These agents may act directly in decreasing activity at the posterior cut surface which inhibits head formation, or they may act by penetrating and blocking any excitation passing forward from the posterior point of origin. It has been found in the experiments on rate of eyespot formation that carbonate-free well water with low pH (Table X) retards, to a certain extent, the whole reconstitutional process, while it increases head frequency. (Child, 1916, observed this same phenomenon with dilute solutions of KCN.) Because of this fact, it seems more logical to assume that the inhibition of the factor inhibiting head development is one aspect of a general inhibiting action which is more effective in decreasing or eliminating that factor, perhaps by block, than in inhibiting the head-forming cells.

*The effects of carbon dioxide.*—It has been seen from the experiments on head frequency that $CO_2$ and the H-ion behave quite differently in the alteration of head frequency even though the end results may be of like nature. It appears as if $CO_2$ penetrates and produces its inhibiting effects quite independently of external pH. It has been found (Osterhout and Dorcas, 1925) that in *Valonia* little or no $CO_2$ enters normal cells except in the form of undissociated molecules. Since HCl ionizes completely in the concentrations used and $H_2CO_3$ is only partly ionized, it seems probable that at least some of the differences in the effects observed are related to the differences in penetration of $CO_2$ and the H-ion. Hyman (1925) has found that $CO_2$ depresses considerably the oxygen consumption in *Euplanaria*. Haywood (1927), in describing $CO_2$ as a narcotic agent, has shown that high concentrations almost completely suppress cleavage in the eggs of *Arbacia*. Jacobs (1912, 1920*a*, 1920*b*, 1922) has shown that the action of $CO_2$ is apparently independent of the

H-ion and that $H_2CO_3$ differs from the strong acids in its ability to penetrate living cells. Its action has been described by this author as being more or less specific. He found that $CO_2$ produces first a liquefaction, followed by coagulation of the protoplasm of *Spirogyra.* Fox (1932, 1933*a*, 1933*b*) goes into the subject of $CO_2$ effects, particularly on the streaming of protoplasm, and attributes the phenomena observed to $CO_2$ narcosis rather than a H-ion effect. In considering the subject of $CO_2$ versus H-ion, Heilbrunn (1928, p. 188) points out that $CO_2$ in solution is more or less anesthetic in action, has a lower surface tension than the pure solvent, and may act as a fat solvent in decreasing protoplasmic viscosity.

Rustia (1925) found that HCl added to well water increased the number of bipolar forms arising from short transverse pieces of *Euplanaria.* He also observed an increase in head frequency with this treatment. As he did not remove the bicarbonates from the well water, it is probable that his results are due to $CO_2$ rather than the H-ion.

In the experiments with $CO_2$ on head frequency described in this paper, it has been shown that $CO_2$ is effective over a wide range of concentrations and that the effect does not depend on the external H-ion. It has also been shown that worms submitted gradually to high concentrations of $CO_2$ will acquire a tolerance to solutions toxic to worms not so treated. It appears, in view of the previous work done on $CO_2$, that all processes pertaining to growth are retarded but that the inhibitory factor arising from the posterior cut surface is affected to a greater extent than the head-forming cells. With this condition existing, it is clearly seen how short transverse pieces of *Euplanaria* which normally give a low head frequency may give increased head frequency in $CO_2$.

*The effects of hydrogen ion.*—In our experiments with carbonate-free water it has been possible to demonstrate the actual effect of H-ion on reconstitution in *Euplanaria.* It has been shown, in the experiments on rate of eyespot formation, that the H-ion in the proper concentration retards, to a measurable extent, reconstitutional development. In concentrations below a critical value (pH 4.3) there is apparently little or no visible effect of the H-ion, while in higher concentrations (pH 4.1 or below) death results. This effect of

the H-ion is doubtless due to the impermeability of living membranes to low concentrations of the ions of strong acids.

Osterhout (1914), in measuring the permeability of tissues of *Laminaria saccharina* by means of their electrical resistance, found that HCl produced a rapid decrease of permeability, which was shortly followed by a rapid increase, which continued until the death-point was reached. In the light of these and other experiments it appears probable that the H-ion does not enter the planarian tissue below a certain concentration (pH 4.3) because of permeability factors. Through a short range of higher concentrations the permeability of the tissues for the H-ion increases, and this results in an increase in the internal acidity. This fall of internal pH may result first in a decreased viscosity of the protoplasm (Heilbrunn, 1928). With a further decrease in pH, coagulation of the protoplasm occurs. It seems extremely likely that metabolic processes are affected by changes in internal pH.[4]

Jewell (1920), in her work on environment and regeneration, found that certain concentrations of HCl in distilled water retarded $CO_2$ production and regenerative growth in tadpoles. In Table 6 of her paper she records a pH of 5.4 as being inhibitory to these processes. She reports a concentration of N/0.00025 HCl as having a pH of 5.4; *N*/0.0005 HCl, a pH of 3.8; and *N*/0.00075 HCl, a pH of 3.2. From these figures it appears that some other factor besides pH is concerned, especially in solutions of pH 5.4. Hyman (1925) found that strong acids in carbonate-free well water had absolutely no effect on rate of oxygen consumption in planarians between pH 7.5 and 5.0. A slight depression (about 15 per cent) was found at pH 4.0. Heilbrunn (1928) suggests that the H-ion may liberate $CO_2$ from protoplasmic buffers and therefore act in much the same way as the direct penetration of $CO_2$. This suggestion is invalidated, at least in part, by Barth (1929), who shows that a number of strong and weak acids will produce coagulation in *Arbacia* eggs at the same external pH if the permeability factor is removed with NaCl. (At the same pH the different acids would be in different concentrations. The

[4] It is apparent that $CO_2$ also alters the internal pH, and indeed it seems probable that these pH changes may be equally as important as the narcotic action of $CO_2$ in altering reconstitutional development.

amount of $CO_2$ liberated depends on the concentration of the acid rather than on the pH.)

In the case of the H-ion it is apparent that the head frequency in *Euplanaria* is increased by a greater inhibition of the effect of the posterior cut than of the head-forming cells. It is also of much interest that the anterior levels of the first zooid are retarded in rate of growth to a greater extent than the more posterior levels of the same zooid. As the anterior levels are originally more active, this greater retardation is a good example of differential inhibition, a phenomenon that has been noted so often in this laboratory.

*Calcium antagonism to the hydrogen ion.*—It has been shown in these experiments that certain effective concentrations of the H-ion can be almost completely antagonized, as regards head frequency, by increasing the calcium concentration of the solution. It is known that calcium is used by living cells in the formation of such structures as the plasma membrane. Salts like sodium and potassium increase the permeability and break down cell membranes. The antagonistic action of calcium on sodium and potassium is too well known to need mentioning. It seems in these experiments that calcium antagonizes the activity of the H-ion by preventing the increase in permeability of the cell membranes which accompanies high concentrations of the H-ion. It is also possible that the antagonistic action of calcium on H-ion may be accomplished to some extent by the different actions of these agents on the protoplasmic colloids.

*The experimental alteration of physiological condition.*—It has been seen throughout these experiments that different lots of animals of the same size often have quite different head frequencies under the same experimental conditions. It appears probable that animals giving a high head frequency at the more posterior levels of the anterior zooid have less axial differential than those giving low head frequencies. However, since head frequency depends on two antagonistic factors (the capacity of the cells concerned in head formation to react to conditions resulting from anterior section, and the factor inhibiting activation and dedifferentiation of these cells which originates at the posterior cut surface), it is evident that the relation between the head-frequency gradient and the general axial differential is not simple and direct. The condition of the head-forming cells and

the effectiveness of the inhibiting factor may vary and can be altered experimentally in either direction, and head frequency in any given case depends on the relation between them. The differences in time of eyespot appearance at different body-levels (Table X) indicate, first, a graded difference in condition of the head-forming cells at different levels, which is apparently a direct expression of the general axial differential, and, second, variation in the amount of this difference in different stocks and under different experimental conditions, which suggests differences in steepness of the general gradient. It is not yet known, however, whether the factor inhibiting head development shows similar graded differences. In the light of the facts at present available, the head-frequency gradient appears to be an indirect and complex expression of the axial gradient involving two opposing and variable factors.

The object of the experiments on conditioning to $CO_2$ was to alter, if possible, the relation between these two factors, and so to alter head frequency. As is evident from Table IX and Figure 5, the head frequencies of conditioned animals are in all cases higher at the more posterior levels than in the non-conditioned; but there is no significant alteration in head frequency at anterior levels. It seems probable in the light of these results that the chief—perhaps the only—effect of the conditioning is the inhibition of the factor inhibiting head development at the more posterior levels. Since this inhibiting factor is apparently chiefly or wholly nervous in character, the conditioning in this case probably consists chiefly in the narcotic action of $CO_2$. The conditioned animals behave as if partially narcotized. They show decreased motor co-ordination and frequently undergo fission, as they do in other narcotics. Certainly the conditioning to $CO_2$ has little or no effect on the head-forming cells, even at the most susceptible anterior levels, for there is no decrease in head frequency there; and, since the inhibiting factor is not effective at anterior levels in 1/8th pieces, there is no increase. Obviously, head frequency can be altered at the more posterior levels by conditioning to $CO_2$ before sectioning, but the data indicate that the effect is chiefly on the nervous system rather than on the general axial gradient. The very great increase in head frequency in animals conditioned to $CO_2$ and subjected to H-ion after section (Fig. 5 *M'*) evidently results from the

combined effect of $CO_2$ in the protoplasm and the H-ion administered. The combined effect of the two agents may provide a more effective block against the inhibitory factor, but it does not necessarily follow that their effects are directly additive physiologically. If this were the case, an increase in percentage of deaths over the non-conditioned lot would be expected, since both $CO_2$ and H-ion are near the lethal concentration; but such increase does not appear.

The data on time of appearance of eyespots (Table X) suggests that some degree of leveling out of the general axial gradient does occur in the H-ion concentration used. The increase in time in H-ion is greater at the anterior level ($X$) than at the posterior level ($Y$) in both lots; and the difference in time between $X$ and $Y$ is less in the experimental than in the control lots. The pieces used in these experiments are long enough to give 100 per cent normal heads. Consequently, the times of appearance of eyespots are regarded as indicating differences in condition of the cells concerned in head formation at different levels.

## SUMMARY

*Euplanaria dorotocephala*, 14–16 mm. in length, were cut into short transverse pieces (approximately 1/8th the length of the animal, exclusive of the head). Only the first four pieces ($A$, $B$, $C$, $D$), which make up most of the anterior zooid, were used. These pieces were caused to reconstitute under various experimental conditions. Upon reconstitution the head frequencies and the number dead at each level were recorded. All experimental lots were controlled with similar lots in well water.

1. Lots of reconstituted worms from animals taken directly from well water, sectioned, and permitted to reconstitute in $CO_2$ solution (pH 5.4–5.6) show an increased head frequency in levels $B$, $C$, and $D$. The death frequencies were greater in pieces $A$ and $D$.

2. Intact animals submitted to high concentrations of $CO_2$ for a few days will become acclimated to $CO_2$ and be able to withstand considerably higher concentrations or concentrations which are lethal to animals that have not been previously treated with this reagent.

3. The effects of $CO_2$ are not due to the external pH, as experiments with carbonate-free well water show that the H-ion has little

or no effect on reconstitution in *Euplanaria* at the pH values of the $CO_2$ solution.

4. Head-frequency experiments in which pieces reconstituted in carbonate-free well water, pH 4.15–4.30, indicate that this concentration of the H-ion induces an increase in head frequency at levels *B*, *C*, and *D*. As in the case of $CO_2$, the death frequencies are greater in *A* and *D* pieces.

5. When $CaCl_2$ (0.5 gm. per liter) is added to carbonate-free well water, pH 4.15, the effect of the H-ion in increasing head frequency is almost completely antagonized. Animals are also protected from the lethal effects of the H-ion by the addition of calcium to the test solution.

6. Stocks of worms from different localities and different lots of worms from the same locality (collected at different times) may yield very different head frequencies under the same experimental conditions. Worms from the same locality and collected at the same time may vary as regards head frequency, depending on the physiological condition of the stock at the time of section and upon the length of time the worms have been kept in the laboratory.

7. Worms have been experimentally conditioned by submitting them to high concentrations of $CO_2$ and H-ion for several days before section. These conditioned worms react quite differently, as regards head frequency, than do non-conditioned worms from the same stock. The chief difference is an increased head frequency in the conditioned worms. Carbon dioxide is a much more effective agent for conditioning than H-ion.

8. In experiments on rate of eyespot formation at different body-levels in *Euplanaria* it was found that 5/16-*X* pieces (anterior 5/16ths of animal exclusive of the head) developed eyespots before 5/16-*Y* pieces (middle 5/16ths of animal). In carbonate-free well water (pH 4.15–4.20) the time of eyespot formation was longer at both levels, but *X* was retarded to a greater extent than *Y*.

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# GONAD DIFFERENTIATION IN THE CHICK EMBRYO AS STUDIED IN HETEROSEXUAL GRAFT AND HOST-GRAFT COMBINATIONS[1]

(Two plates)

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LILLIE (1917) reported that the embryonic sex glands of the female member of a pair of opposite-sexed twin calves are modified in the male direction as the result of a fusion of its chorionic blood vessels with those of its male co-twin. Several investigators have since employed the chorioallantoic grafting technique as a means of incorporating two gonads of opposite sex in the same embryonic blood circulation of the chick embryo. Such a host-graft relationship would seem to be a suitable method of testing the influence of a gonad graft upon the reproductive organs of the host embryo, or of the gonads of the host upon the engrafted gonad.

Previous experiments involving the transplantation of gonad tissue to the chorioallantoic membrane have been of two general types: (1) Gonad rudiments were transplanted to host embryos whose sex glands were already morphologically differentiated to determine what influence the sex of the host might have upon the capacity of the rudiment to produce a gonad of specific sex (Willier, 1925 and 1927, and Corinaldesi, 1927*a* and 1927*b*). (2) Sexually differentiated gonad tissue isolated from embryos and from juvenile or adult fowl were transplanted to host embryos whose sex glands were in an early stage of morphological sex differentiation (7–9 days' incubation). The latter type of experiment was carried out by Minoura (1921), Greenwood (1925), Kemp (1925 and 1927), and Willier (1927) to determine whether or not the gonad graft influences the process of differentiation in the sex glands and ducts of the host. With the exception of those reported by Minoura, these experiments were negative on the question of sex hormone action in the chick.

[1] This work was carried out at the suggestion and under the direction of Dr. B. H. Willier, to whom I am greatly indebted for helpful suggestions and valuable aid.

Consequently, a further study, employing different methods of approach, seemed advisable.

The first series of experiments reported here was designed to test the self-differentiating capacity of the morphologically undifferentiated gonad when associated for 8 or 9 days in the same chorio-allantoic graft with differentiated gonad tissue (isolated from birds ranging in age between 12 days' incubation and $3\frac{1}{2}$ months' post-hatching). This type of experiment enables one to test possible effects of contiguous or closely associated cortical and testicular tissues upon the direction of differentiation of the indifferent gonad rudiment. Such a relationship may be of primary importance in influencing the course of sex differentiation, as Witschi (1931 and 1934) has suggested for amphibians.

Certain combination grafts in this series contain gonad tissue known to be producing hormone at the time of implantation, i.e., from hatched chicks. Such grafts should throw some light on the question of the capacity of sex hormones which condition secondary sexual characteristics to function also as sex-differentiating hormones.

The second group of experiments was performed to ascertain what influence gonads, already structurally differentiated as to sex, and of the same age, may exert upon each other in heterosexual combination in the same graft. Testes and ovaries were isolated from donors of approximately 18 days of incubation and transplanted in heterosexual pairs to a host embryo, where they developed in close proximity or in direct contact with one another for a period of 8 or 9 days.

The third set of experiments was performed in an endeavor to elicit a response on the part of the reproductive organs of the host embryo to large amounts of gonad tissue transplanted to the chorio-allantoic membrane. So-called "multiple-testis" grafts were made in an attempt to effect in the host embryo a high dosage of male hormone, thereby possibly inducing modification of its reproductive organs by increasing the stimulus above a certain threshold.

## MATERIAL AND METHODS

Donor material composing the grafts consisted of: (1) chick gonads morphologically undifferentiated as to sex, and (2) morphologically differentiated gonad tissue. The undifferentiated gonad

rudiments were obtained by isolating separately the right and left urinogenital ridges from embryos incubated between 4½ and 6 days. This operation was performed under sterile conditions in a Petri dish containing warm physiological saline. The differentiated gonad tissue was secured from embryos during the latter half of their incubation period or from hatched birds as old as 3½ months. Immediately after removal, this tissue was placed into warm, sterile salt solution. The grafting technique was essentially the same as that described by Willier (1924 and 1927). The tissue or tissues chosen to compose the graft were removed from the salt solution on the point of a glass needle and placed at the junction of two blood vessels on the chorioallantoic membrane of a previously prepared 8- or 9-day host embryo.

When a right gonad rudiment was combined with differentiated gonad tissue in a combination graft, the left one was transplanted alone to the membrane of a second host, thus serving as control; and vice versa. According to Willier (1927), the morphologically indifferent gonad has the capacity to self-differentiate according to its original plan of organization regardless of the sex of its host. Such a control graft thus affords a means of ascertaining the originally determined sex of its partner in the combination graft.

Eight or 9 days after operation the grafts were removed, fixed in Bouin's fluid, sectioned at 7 $\mu$ and stained with iron haematoxylin. The reproductive organs of all hosts were closely examined under a binocular microscope for evidence of any atypical structure. The ovaries of female hosts bearing "multiple-testis" grafts were examined histologically. A number of control grafts of testicular or ovarian tissue, as well as normal ovaries and testes of an age corresponding to those contained in the combination grafts, were frequently referred to during the examination of the experimental material. Eggs and tissues from the white Leghorn breed of fowl were used exclusively in these experiments.

## ASSOCIATION OF AN INDIFFERENT GONAD RUDIMENT WITH DIFFERENTIATED GONAD TISSUE

### A. THEORETICAL NUMBER OF GONAD COMBINATIONS

At the time the structurally indifferent gonad primordium (4–6 days' incubation) is transplanted with older testicular or ovarian tissue, only its age and laterality are known. During the 8 or 9 days

on the membrane the rudiment undergoes sex differentiation. To test its self-differentiating capacity in the presence of older gonad tissue, eight types of double transplants were made: right or left gonad rudiments, structurally undifferentiated as to sex, were transplanted with a piece of a right or left ovary or a right or left testis. The control in each case was the opposite gonad rudiment transplanted alone to a second host.

If the structurally undifferentiated gonad has the capacity to self-differentiate in accordance with its originally determined sex, and the laterality of each component of the graft is considered, sixteen possible combinations of gonad tissue should arise from the eight types of double transplants. Considering the marked and significant difference in structure and function between the left and the right ovary in the domestic fowl, it was essential to record the laterality of all ovarian tissue transplanted. Since, on the other hand, no such differences exist between the right and left testis in late embryonic, juvenile, or adult birds, the number of possible gonad combination may be reduced to twelve if the laterality of the testicular tissue transplanted is disregarded.

If the sex of the host is considered, twenty-four possible gonad relationships exist, twenty-three of which have been obtained in these experiments. It was found that each of the twelve possible gonad combinations was represented in the combination grafts, following the sex differentiation of the structurally indifferent component. In a number of cases the gonad rudiment which served as a control to its partner in a combination graft was lost. This is to be expected, since death of the host frequently follows the operation. In eighty-three out of the total one hundred and twenty-three cases the gonad rudiment in both the combination and the control graft survived. Table I shows the twelve possible types of combination graft, the number of cases recovered representing each type, and their relation to the sex of the host. Those in which the gonad rudiment gave rise to a gonad-like body, unspecific as to sex, are also recorded.

### B. THE MORPHOLOGY OF THE "INDIFFERENT" GONAD PRIMORDIUM AT THE TIME OF IMPLANTATION

The majority of gonad primordia transplanted in combination grafts were isolated from donor embryos which had been incubated

for 5 or 5½ days. Two were as old as 6 days; fourteen were 4 and a fraction days of age.

At the end of the fourth day of incubation, according to Swift (1915), the developing gonad consists of a whitish ridge approximately 1.5 mm. in length lying on the ventromedial surface of the Wolffian body. It is composed of three types of tissue: (1) a germinal epithelium, two or three cell layers in thickness; (2) a compact mass of embryonic mesenchyme which lies beneath the germinal epithelium; and (3) primordial germ cells, which are found between the epithelial cells of the germinal epithelium, as well as lying deeper in the embryonic mesenchyme. It is an interesting fact that the number of primordial germ cells at this early stage of development is greater in the left gonad rudiment than in the right (Firket, 1914; Swift, 1915).

TABLE I

| Associated Differentiated Gonad | Sex of Host | Right Undifferentiated Gonad | | | Left Undifferentiated Gonad | | | |
|---|---|---|---|---|---|---|---|---|
| | | Right Ovary | Right Testis | Right Not Ascertained | Left Ovary | | Left Testis | Left Not Ascertained |
| | | | | | Typical | Atypical | | |
| Left ovary | ♀ | 3 | 2 | 1 | 4 | 1 | 2 | 0 |
| | ♂ | 2 | 2 | 0 | 1 | 0 | 4 | 1 |
| Right ovary | ♀ | 3 | 1 | 0 | 4 | 0 | 5 | 0 |
| | ♂ | 2 | 0 | 1 | 1 | 3 | 5 | 1 |
| Testis | ♀ | 1 | 4 | 0 | 7 | 1 | 14 | 4 |
| | ♂ | 4 | 2 | 3 | 4 | 14 | 11 | 5 |
| Total | ....... | 15 | 11 | 5 | 21 | 19 | 41 | 11 |

At approximately the 132d hour of incubation the primary sex cords (cords of first proliferation) begin to invaginate into the underlying mesenchyme from the germinal epithelium. This process continues for the next 24 hours, certain of the sex cords in the meantime making connection with certain Malpigian corpuscles of the Wolffian body by means of the rete cords (cords of urinogenital union). At 6½ days (156 hours) the proliferation of the primary sex cords rather

abruptly ceases and sexual differences become morphologically apparent.

In these experiments, therefore, the gonad primordia contributing to the combination grafts or transplanted singly as controls were at the time of implantation "morphologically indifferent" as to sex.

### C. SEX OF GONADS DIFFERENTIATING FROM GRAFTED RUDIMENTS

At 14 or 15 days of incubation the gonad of the chick has a characteristic histological structure which enables one to determine the sex, and, in the case of the female, the laterality. With certain exceptions, which will be described later, the gonad rudiments which gave rise to gonads in combination grafts possess a similar characteristic histological structure.

The typical left ovary found in a combination graft has two markedly different structural components. It possesses both a cortex and a medulla (Plate I, Fig. 3). The medulla is composed of a diffuse network of epithelial cords. The cortex is more compact in appearance and is made up of cords of epithelial and germ cells which have invaginated from the germinal epithelium, at this time still composed of columnar epithelial cells and a few germ cells. This cortical layer overlies the medulla and covers the entire surface of the ovary with the exception of the area where the gland is attached to the Wolffian body. In short, the appearance of the ovary is strikingly similar to a normal left ovary of corresponding age.

The right ovary found in a combination graft differs from the typical left ovary in three principal characteristics: (1) its much smaller size; (2) absence of a set of cortical cords; and (3) a flat, inactive epithelial covering (Plate I, Fig. 1).

A testis which arises from a gonad rudiment in a combination graft can be easily distinguished from either the left or the right ovary since it possesses a well-developed tunica albuginea and a set of elongated sex cords whose structural components—the sex cells and supporting epithelial cells—are arranged in a characteristic spacial relationship within the cord (Plate I, Fig. 2). The identification of sex of all gonads arising from transplanted "indifferent" gonad rudiments was made on the basis of the above-mentioned criteria.

## PLATE I

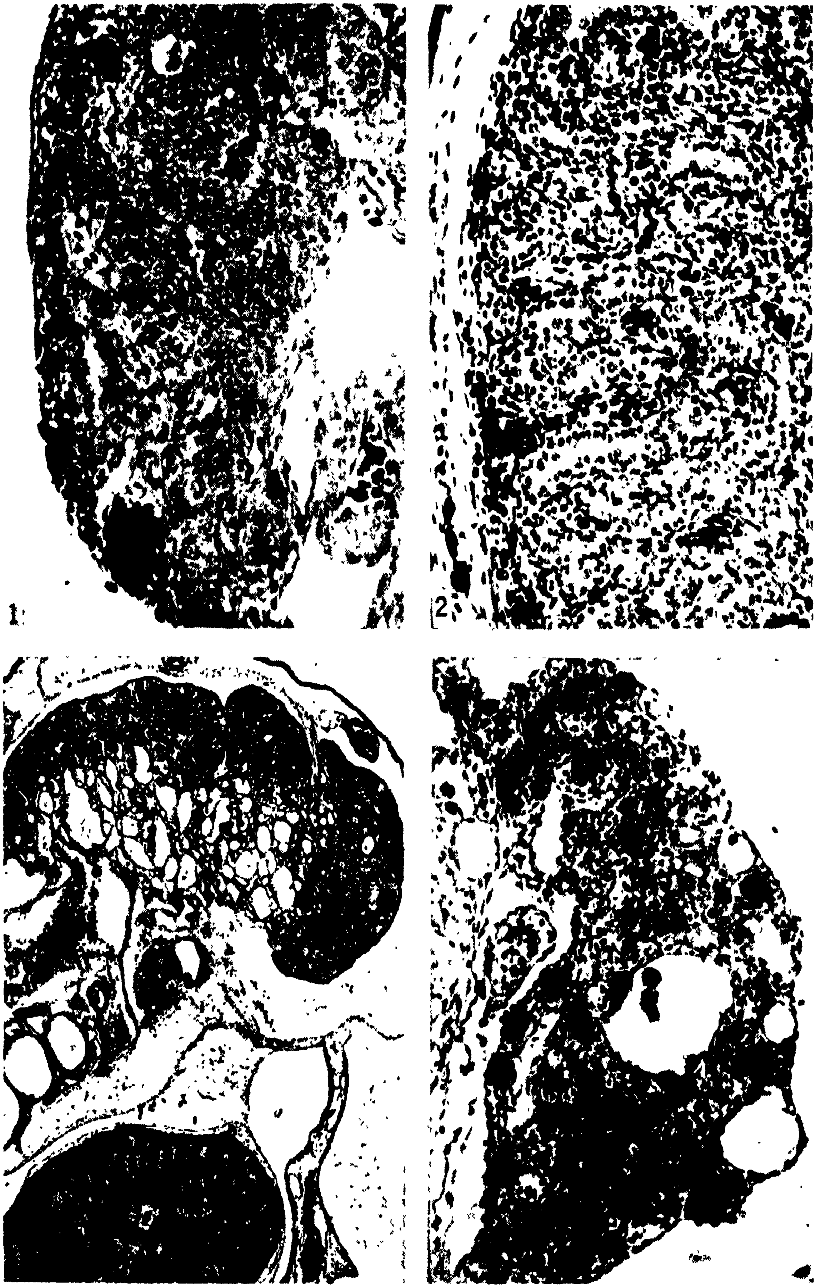
FIG. 1.—Section through a right ovary which differentiated from a right gonad rudiment combined with testicular tissue from a 51-day-old cock; grown for 9 days on a male host. ×500.

FIG. 2.—Section through a testis derived from a right gonad rudiment combined with a left ovary from an embryo of 12½ days' incubation and grown on a female host. ×400.

FIG. 3.—Longitudinal section through a left ovary differentiated from a left gonad rudiment in combination with a 17-day embryonic testis; 9 days on a male host. Note well-differentiated medulla and cortex. ×65.

FIG. 4.—Section of a modified left ovary derived from a left gonad rudiment, combined with an 18-day right ovary and grown on a male host. ×315.

PLATE I



The older gonad tissue which was transplanted with the sexually undifferentiated rudiment is strikingly different from the young sex gland which has undergone differentiation during the growth period of the graft. The difference in age is associated with sharp histological differences in structure, thus facilitating identification of the two components. Even in those cases where the sex of the younger gonad and the older gonad tissue is the same, no opportunity for confusing the two exists.

### D. DIFFERENTIATION OF THE RIGHT "INDIFFERENT" GONAD PRIMORDIUM

Thirty-one grafts were examined in which a right "indifferent" gonad rudiment was combined with ovarian or testicular tissue. Fifteen differentiated into right ovaries, eleven into testes, and five into gonad-like bodies whose sex could not be ascertained (see Table I).

1. *Right ovaries.*—Five right ovaries differentiated in grafts with left ovarian tissue (from embryos between 13 and 19 days' incubation), three of which developed on female and two on male hosts. Five were combined with right ovaries from embryos of 18 days' incubation, three of which developed on female and two on male hosts. Of the remaining five, which developed with testicular tissue from chicks ranging in age between 17 days' incubation and 80 days' post-hatching, four developed on male, and one on a female, host.

Regardless of the nature of the combination, the right ovary, which differentiates from the right indifferent gonad, is small and quite similar to the normal in general appearance. In the greater number of cases it consists of medullary elements only, although occasionally it is found to possess a few scattered areas of cortical tissue. That the presence of these cortical elements is not due to any specific factor of the environment operating during the development of the graft is strongly suggested by their rather frequent appearance in normal right ovaries (Brode, 1928).

In the majority of cases the right ovary is a more or less compact, definitely circumscribed, elongated body, broadly attached to the mesonephros (Plate I, Fig. 1). Occasionally, however, it may have a loose texture and present the appearance of a stromal network. Histologically, it consists of cords of epithelial or primordial germ

cells imbedded in a connective tissue stroma. Near the point where the ovary is attached to the Wolffian body, distention of the medullary cords frequently occurs. In the lumina of such distended cords epithelial cells or primordial germ cells may be found, either singly or in nests consisting of several cells. The surface of the right ovary lacks a distinct germinal epithelium and is covered by a single layer of flattened cells. Even in those right ovaries in which scattered cortical elements are present, the overlying germinal epithelium is flattened and inactive. This inactive condition of the germinal epithelium is also characteristic of the normal right ovary (Firket, 1920).

On theoretical grounds it is expected that the right ovary, known to possess male potentialities, might be modified in the male direction under the stimulation of hormones from older testicular tissue. In five cases right ovaries differentiated from right gonad rudiments which grew in the same graft with testicular tissue obtained from donors ranging in age from 17 days' incubation to 80 days' post-hatching. Four of these grafts grew on male hosts; one, on a female host. That these gonad rudiments were really determined as right ovaries is shown by the fact that, in the three cases in which the partner left gonad rudiment survived, it differentiated into a left ovary. Although there are slight variations in the structure of the right ovaries, they are within the range found characteristic for the normal right ovary. Furthermore, they differentiate equally well when combined with a testis and grown on a male host as when combined with ovarian tissue and grown on a female host.

2. *Right testes.*—Four of the eleven testes which differentiated from right gonad rudiments were combined with left ovarian tissue from donors ranging in age between 13 days' incubation and 75 days' post-hatching. Two grew on male, and two on female, hosts. One testis was combined with a right ovary from an embryo of 17½ days' incubation and grew on a female host, and the remaining six were associated with testicular tissue from embryos of 18 days' incubation or from chicks as old as 80 days. Four of these developed on female, and two on male, hosts.

Testes which arise from right gonad rudiments in combination grafts are similar in structure to those arising in grafts of right rudi-

ments transplanted alone, and closely resemble those described by Willier (1927). Their essential structural components consist of a surrounding tunica albuginea which is continuous with the connective tissue stroma containing the sexual cords. These cords are composed of supporting epithelial cells and primordial germ cells.

3. *Right gonad-like bodies.*—Although the right gonad rudiment differentiates in the majority of combination grafts into gonads of specific sex, in five such grafts it gave rise to gonad-like bodies whose sex could not be definitely ascertained. These vary considerably in structure. They are always small and generally poorly organized. Some have a definite shape and are rather sharply delimited from other structures in the graft, in which case they present a compact appearance and consist of "nests" of epithelial cells and germ cells imbedded in supporting connective tissue. Others can be considered little more than scattered groups of epithelial and primordial germ cells which occur in the mesenchyme of the graft in the vicinity of the mesonephric body.

It is necessary to investigate the conditions under which these right gonad-like bodies develop, to determine whether their origin may be related to the sex of the associated gonad or to the sex of the host.

Of the five, one was combined with a right ovary from an embryo of 15 days' incubation and grown on a male host; three with a pair of testes from 16-day embryos, on male hosts; and one with left ovarian tissue from an 80-day-old hen on a female host. Unfortunately, four of the five left gonad rudiments which served as controls for these grafts were lost, because of death of the host, and the originally determined sex of four of the five right rudiments in the combination grafts was therefore not ascertained. In the fifth case the right rudiment, which failed to produce a gonad of specific sex, had a female-determining constitution. It was associated with two testes from a 16-day embryo and grew on a male host. Little direct evidence is available, therefore, which points to a positive correlation between the sex of the associated gonad and the failure of the rudiment to differentiate specifically as to sex. Certain indirect evidence, however, indicates that the origin of these right gonad-like bodies is not due to sex hormones secreted by either the associated gonad or the

sex glands of the host. (1) Right gonad rudiments, originally determined female, develop into typical right ovaries when associated in the same graft with testicular tissue and grown on male hosts (see Plate I, Fig. 1). (2) Testes having a normal appearance arise from right gonad rudiments transplanted with left ovarian tissue and grown on female hosts (see Plate I, Fig. 2). (3) Four morphologically "indifferent" right gonads which were transplanted alone as controls developed into gonad-like bodies of undetermined sex, similar in structure to those occurring in the combination grafts. Of these four cases, two developed on male, and two on female, hosts.

### E. DIFFERENTIATION OF THE LEFT "INDIFFERENT" GONAD

Ninety-two grafts were obtained in which a morphologically undifferentiated left gonad developed in combination with older ovarian or testicular tissue. Forty-one differentiated into left testes and forty into left ovaries. The remaining eleven gave rise to gonad-like structures which are undistinguishable as to sex.

1. *Left testes.*—Of the forty-one testes, six were combined with left ovarian tissue obtained from donors ranging in age between 18 days' incubation and 80 days' post-hatching. Two grew on female, and four on male, hosts. Ten were associated with right ovaries from embryos incubated 12½–18 days. Of these, five developed on female, and five on male, hosts. Twenty-five developed in grafts with testicular tissue from donors ranging in age between 12½ days' incubation and 51 days' post-hatching. Of these, fourteen grew on female hosts, the remaining eleven on male hosts.

Twenty-five of the forty-one right rudiments which served as controls to the left rudiments in combination grafts survived, each of which differentiated into a testis, thus proving that their partners in the combination grafts differentiated according to their originally determined male constitution.

The testes derived from left gonad primordia in combination grafts are similar in every respect to those already described which differentiated from right gonad rudiments grown under similar conditions. Moreover, they are identical in histological structure to those arising from either left or right gonad rudiments which were transplanted alone to the chorioallantoic membrane.

2. *Left ovaries.*—Left gonad rudiments, whose originally determined sex is female, exhibit wide variation in their capacity for self-differentiation in combination grafts. Of the forty left ovaries, twenty-one are structurally similar to the normal left ovary of a 13- or 14-day chick embryo, and nineteen show structural abnormalities in varying degrees.

Of the twenty-one which show a typical left ovarian structure, five developed in grafts with older left ovarian tissue obtained from embryos of 14–18 days' incubation and from hatched fowl as old as 112 days. Four such grafts developed on female, and one on a male, host. Five developed in combination with right ovaries from 13- to 18-day chick embryos; four on female, and one on a male, host. Eleven developed in grafts with testicular tissue (donors ranging in age from 16 days' incubation to 48 days after hatching), seven of which developed on female, and four on male, hosts.

In these twenty-one cases the ovary is composed of well-differentiated cortical and medullary elements (Plate I, Fig. 3), both of which contain epithelial and germ cells. A few cortical cords are in the process of invagination and remain attached to the well-developed columnar germinal epithelium, while others have lost connection with their point of origin. The medulla shows an outer compact area and an inner area composed of much-distended cords. Such grafted left ovaries have been described in detail by Willier (1927). The histological picture is remarkably similar to that of a normal left ovary of the same age.

Although left ovaries closely resembling the normal may develop from left "indifferent" gonads in combination grafts, nineteen left ovaries developing from left gonad rudiments in similar grafts show rather striking deviations from the normal structure.

Of these nineteen, one was associated with left ovarian tissue from an 80-day-old hen and grown on a female host. Three were combined with right ovaries from 18-day embryos and grown on male hosts. The remaining fifteen were combined with testicular tissue from donors ranging in age between 16 days' incubation and 48 days' post-hatching. Fourteen of these developed on male, and one on a female, host. Sixteen of the nineteen control right gonads survived and differentiated into right ovaries, thus proving that their

partner left rudiments in the combination grafts were originally determined as female. These atypically formed left female sex glands have certain characteristics in common: (1) small size; (2) absence of a columnar-celled germinal epithelium; (3) reduction or absence of cortical cords; and (4) compact masses of cellular cords, apparently medullary, which contain a greater number of germ cells than is characteristic of the medulla of normal left ovaries of corresponding age. In general, their appearance is similar to that of right ovaries derived from the opposite right "indifferent" gonad transplanted alone as a control.

The atypical left ovaries show individual variation in structure, and therefore a number of them will be described separately. One which was combined with left ovarian tissue from an 80-day-old hen and grown on a female host shows, through four or five serial sections, a germinal epithelium composed of columnar cells from which one or two cortical cords have invaginated. The greater portion of its surface, however, is devoid of a typical germinal epithelium and is covered by a layer of flattened epithelial cells beneath which no cortical elements have been formed. The medulla, which composes nearly the entire gland, consists of a connective tissue stroma in which are imbedded the medullary cords. Primordial germ cells are abundant, typically arranged in "nests." That the gonad's originally determined sex was female is proved by the fact that its partner right gonad, transplanted alone, developed into a typical right ovary.

One of the three modified left ovaries which were combined with right ovaries obtained from chicks near hatching and grown on male hosts is illustrated in Plate I, Figure 4. The germinal epithelium is composed of flattened cells, and cortical cords are completely lacking. Many primordial germ cells, arranged in "nests," are present. Two or three open spaces, bounded by a layer of flattened epithelial cells, suggest a distention of medullary cords. In some of these open spaces are found primordial germ cells, either singly or in small groups. The ovary has, in general, a compact appearance and is found to consist apparently of medullary elements only. Unfortunately, the control right gonad was lost, owing to death of the host following operation. However, its structure so closely corresponds to other

atypical left ovaries that little doubt exists that the left rudiment which gave rise to it was originally determined female.

Of the fifteen atypical left ovaries which developed in combination with testicular tissue, three may be described to illustrate the range of variation in structure which they exhibit. A left gonad rudiment combined with a testis from an 18-day embryo and grown on a male host gave rise to the left ovary illustrated in Plate II, Figure 5. The gland is small and covered by a distinct germinal epithelium composed of cuboidal epithelial cells and a few germ cells. From this germinal epithelium several cellular cords have invaginated which are probably cortical cords, although they are more slender and contain fewer well-differentiated germ cells than do normal cortical cords. The deeper portion of the gland is composed of nests of cells which contain both epithelial and primordial germ cells. These structures probably represent the medullary component of the ovary.

The left female sex gland, shown in Plate II, Figure 6, developed in a graft with two testes obtained from a 17-day embryo. It was grown on a male host. It has a germinal epithelium covering only about one-third of its free surface, from which no cortical cords have invaginated. The remainder of the gland is comprised of medullary cords imbedded in a connective-tissue stroma. These medullary cords are made up of epithelial cells and a few primordial germ cells.

A left ovary which is extremely abnormal in structure was derived from an "indifferent" left gonad primordium combined with a testis from an 18-day chick embryo and grown on a male host (Plate II, Fig. 7). It is small and shows a germinal epithelium composed of flattened cells; no cortical cords are present. The gland is composed entirely of medullary cords which are made up of two types of cells, epithelial and primordial germ cells, the latter greatly predominating. This fact gives the gland the appearance, when examined under low magnification, of being composed almost entirely of "nests" of primordial germ cells. No distention of the medullary cords occurs in this left ovary. The control graft contains a well-differentiated right ovary.

It is evident, then, that left ovaries arising from "indifferent" left gonad rudiments combined with testicular tissue may appear quite

## PLATE II

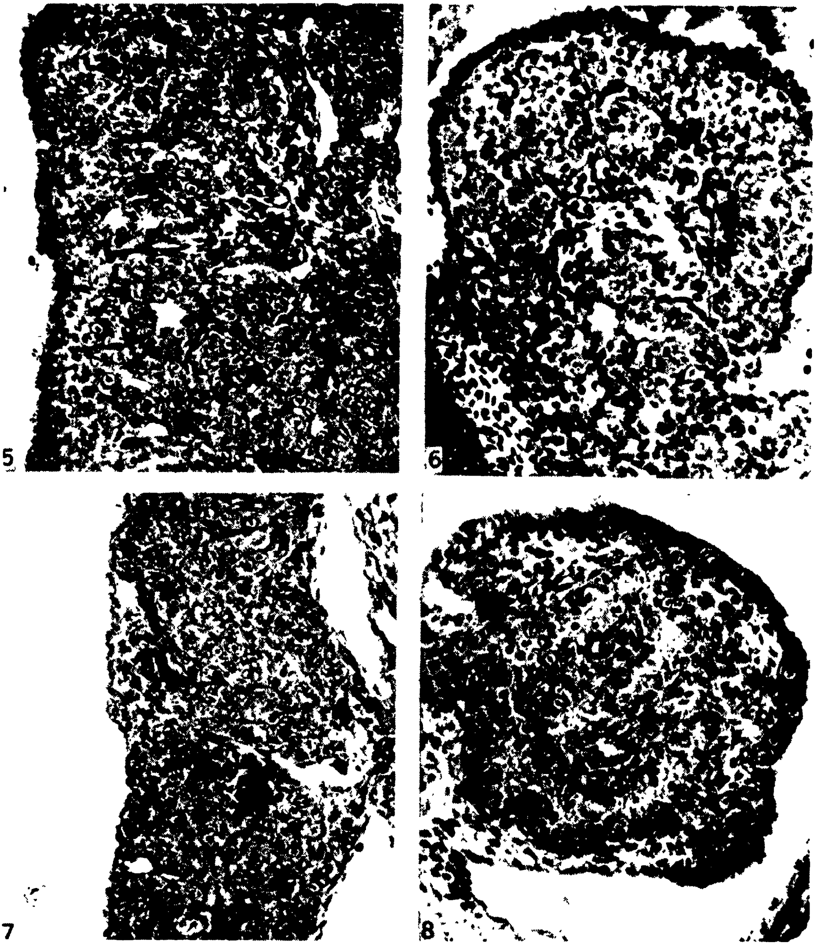
FIG. 5.—Section of a slightly modified left ovary which developed in the same graft with an 18-day embryonic testis. The host was a male. $\times 500$.

FIG. 6.—Section of a modified left ovary derived from a left gonad rudiment which was associated with two testes obtained from a 17-day embryo; male host. $\times 525$.

FIG. 7.—Section of an abnormal left ovary which developed from a left rudiment combined with an 18-day testis and grown on a male host. $\times 500$.

FIG. 8.—Section of a gonad-like body possessing male sex cords and a germinal epithelium. It arose from a left gonad rudiment combined with a right ovary of a 16-day embryo. The host was a male. $\times 525$.

PLATE II



normal or may show structural modification in varying degrees. In those less strikingly atypical in structure a germinal epithelium may be present from which a very sparse proliferation of cortical cords has occurred (one case); those showing greater modification may possess a germinal epithelium consisting of columnar or cuboidal cells but no cortical cords (two cases); finally, the left ovary may consist of medullary elements only, both germinal epithelium and cortical cords being entirely lacking (twelve cases). This tendency for left gonad rudiments having a female-determining constitution to develop in an atypical manner in combination grafts stands out in marked contrast to the capacity for typical differentiation exhibited by right rudiments which were originally determined female and by rudiments having a male-determining constitution (either right or left). It will be noted in Table I that, of the seventeen left ovaries which differentiated in combination grafts grown on female hosts, fifteen possess a typical structure and two are atypical. Of the twenty-three left ovaries in combination grafts supported by male hosts, only six are typical in structure, while seventeen present an atypical histological picture. These data, examined statistically, give a difference of 62.1 per cent, or 3.88 times the standard error. Only 12.5 per cent of the left ovaries which differentiated in grafts containing testicular tissue and which grew on female hosts were abnormal, while similar left ovaries also associated with testicular tissue but grown on male hosts present an atypical histological picture in 77.7 per cent of the cases. It would appear, therefore, that even though the sex of the differentiated gonad tissue in the same graft is disregarded, the sex of the host must be considered a possible factor influencing the capacity of the left rudiment, originally determined female, to differentiate into a typical left ovary.

3. *Left gonad-like bodies.*—Of the eleven gonad-like bodies derived from left gonad rudiments, one was combined with a left ovary from a 19-day embryo and grown on a male host. Another developed, with a 16-day embryonic right ovary, on a male host; and nine were found in grafts with testicular tissue from donors ranging in age between 14 days' incubation and 80 days' post-hatching. Of these nine, four developed on female, and five on male, hosts.

With one exception, these gonad-like bodies are very similar in structure to those which develop from right gonad rudiments. The one exceptional left gonad, which developed in a graft with a right ovary, on a male host, has definite structural peculiarities (Plate II, Fig. 8). Most of its free surface is covered with a germinal epithelium composed of columnar cells between which occur primordial germ cells. The greater portion of the gland is composed of well-differentiated sex cords which closely resemble, structurally, those of a normal testis of a chick of approximately 14 days' incubation. The primordial germ cells are distributed irregularly throughout the sex cords, while the supporting epithelial cells are arranged radially around the long axis of the cord, their oval nuclei showing a linear arrangement along the periphery. Although the sexual cords are typically testicular in appearance, the germinal epithelium is more characteristic of a left ovary. Unfortunately, the control graft containing the partner right gonad was not recovered.

The originally determined sex of four of these eleven gonad-like bodies was ascertained. These four, originally determined male, were combined with testicular tissue from donors aged 50 days after hatching. Two of them developed on female, and two on male, hosts.

Three similar gonad-like bodies developed from left gonads transplanted alone. Two of these grafts developed on female, the remaining one on a male, host.

#### F. DEVELOPMENT OF THE OLDER GONAD COMPONENT IN THE GRAFT

Testes isolated from chicks just before, or a few days after, hatching become completely vascularized following transplantation, and continue to develop in essentially a normal manner (Plate I, Fig. 3). Large transplants of testicular tissue from juvenile fowl (40–90 days of age) frequently fail to become completely vascularized rapidly enough to prevent necrosis at their center. Even in those cases where degenerative changes are evident centrally, a large number of apparently healthy and well-differentiated semeniferous tubules are present around the periphery of the transplant.

All right ovaries contributing to combination grafts were isolated from chicks during the last 4 or 5 days of the incubation period. After 8 or 9 days in the graft they are similar in appearance to nor-

mal right ovaries of chicks 4 or 5 days after hatching. They vary in size and shape, but their histological structure remains quite typically right ovarian.

Left ovarian tissue from embryonic or newly hatched chicks becomes rapidly vascularized following transplantation. Donor tissue isolated at 17 or 18 days of incubation shows well-developed Graffian follicles after 9 days on the host. Large pieces of ovarian tissue from young hens (60–112 days of age) may become necrotic centrally, owing to slow and incomplete vascularization following transplantation. The periphery of the transplant always shows many follicles containing ova, some of which have a massive accumulation of yolk, and nuclei containing formed chromosomes.

It can be definitely stated that gonad tissue from late embryonic or hatched fowl, when incorporated into a combination graft, continues to develop in a normal manner, with the exception of the central area of large transplants, which become vascularized too slowly to prevent degenerative changes.

## COMBINATION GRAFTS OF SEXUALLY DIFFERENTIATED GONADS

To ascertain what influence gonads, already structurally differentiated as to sex, and of the same age, may exert upon each other in heterosexual combination in the same graft, testes and ovaries were isolated from donors of 18 days' incubation and transplanted in heterosexual pairs to a host embryo. There they developed in close proximity or in direct contact with one another for a period of 8 or 9 days.

### A. COMBINATION GRAFTS OF TESTIS AND RIGHT OVARY

Seven grafts which resulted from a combination of a testis and right ovary show both gonads to have a structure essentially similar to normal gonads of corresponding age. The testis in each case continued to develop in a normal manner and exhibits a histological picture similar in most respects to the testis of a 5- or 6-day hatched chick. The semeniferous tubules are greatly elongated; and lumina, never present at the time of isolation, have developed in many of them. The right ovaries vary somewhat in structure, but not beyond the range of variation to which normal right ovaries are subject. In three cases the right ovary is a compact, definitely circumscribed

body. In two grafts it has the appearance of a network, and in one case has become infiltrated with lymphocytes. In another graft the right ovary is abnormally large and contains a considerable amount of cortical tissue. The protocol reveals, however, that areas of cortical elements were observed macroscopically on the surface of the ovary at the time of implantation.

### B. COMBINATION GRAFTS OF TESTIS AND LEFT OVARY (NINE CASES)

Six grafts arising from the transplantation of left ovary with testis show both components of the graft to have continued their growth and development with little or no variation from their normal structure. In four of these six cases the testis and ovary are in direct contact, and probably had been so during the 9-day growth period on the host embryo. In the remaining three grafts the testis is quite normal in appearance and closely corresponds to the testis of a chick 5 or 6 days after hatching, but the associated left ovaries show an appreciable thinning of the cortical layer with a reduction in the number of primitive ova contained. Grafts containing normal left ovaries in the presence of testicular tissue are found on both male and female hosts. Of the three combination grafts showing ovaries with reduced cortex, two grew on male hosts and one on a female host. In two instances left ovaries of corresponding age transplanted alone on hosts (one male, one female) have shown a similar thinning of the cortical layer.

## RESPONSE OF THE REPRODUCTIVE ORGANS OF THE HOST TO GONAD GRAFTS

The reproductive organs of all host embryos bearing combination, as well as control, grafts were examined under a binocular dissecting microscope for evidence of a reaction to the various types of gonad grafts. Some variations from the typical anatomical structure were observed in the reproductive organs of the hosts. These include variations in the size and shape of the gonads themselves, particularly the right ovary. The length of the persistent "cloacal stump" of the right oviduct and the diameter of the Wolffian ducts, the left oviduct, and of the shell gland are all subject to slight variations. These variations from the typical structure cannot be considered as

specific responses to gonad grafts, since they are very similar to those reported previously by Willier and Yuh (1928), who proved that they also occurred in host embryos bearing non-gonad grafts. Similar variations were observed in the structure of the reproductive organs of 18-day normally incubated chick embryos whose gonads served as donor tissue in these experiments.

### "MULTIPLE-GONAD" GRAFTS

It seemed possible that lack of response to gonad grafts on the part of the hosts' reproductive organs might be due solely to the failure of the small pieces of transplanted gonad tissue to secrete sufficient sex hormone to bring about a positive response. It was hoped that grafts consisting of four or five 18-day embryonic gonads transplanted to 9-day host embryos might produce sufficient hormone to elicit a response in the reproductive organs of the hosts. Grafts consisting of as many as five testes from 18-day embryos developed for 8 or 9 days on both male and female hosts. The grafts were well vascularized, and the transplanted testicular tissue was apparently in a healthy and normal condition. The reproductive organs of the male hosts bearing such grafts were examined under the binocular dissecting microscope and found to correspond closely to those of a normally incubated 18-day male chick embryo. The sex ducts of the female hosts were normal in appearance, and histological examination of the ovaries of these hosts revealed no deviations from the normal structure. It would seem definitely established, therefore, that even large amounts of testicular tissue, from embryos just prior to hatching, are unable, when transplanted to the chorioallantoic membrane at 9 days' incubation, to affect the reproductive organs of the host.

No grafts resulting from the transplantation of three or more 18-day left ovaries were recovered, since the hosts in all such cases failed to survive the operation. Death of the host under these conditions was probably due to the large volume of tissue transplanted.

### DISCUSSION

The evidence presented in the foregoing sections reveals that, with certain exceptions to be noted below, right or left indifferent gonad

rudiments, in case they have a male-determining constitution, have the capacity for self-differentiation into a testis regardless of the sex of the associated differentiated gonad and of the sex of the host. Either right or left gonad rudiments whose originally determined sex was male differentiated into testes although they were subject to hormones from an associated ovary (left or right) and grown on a female host.

Such a capacity for independent differentiation is likewise characteristic of a right gonad rudiment when it has a female-determining constitution. Such a right rudiment, although associated with testicular tissue of various ages and grown on a male host, has the capacity to differentiate into a typical right ovary.

The failure of the right ovary, developing under these conditions, to show any evidence of inversion is interesting, since it is known to have male potentialities and the capacity to transform into a testis following left ovariectomy of chicks just after hatching (Benoit, 1923*a*, 1923*b*, 1923*c*; Domm, 1927 and 1929; and others). Although the growth period of the right ovary in the combination graft is very short in comparison to the time required for the inversion following ovariectomy, it would be expected that at least the early phase of the process might be detectable histologically.

On the contrary, the left indifferent gonad primordium having a female-determining constitution exhibits considerable variation in its capacity to self-differentiate under similar developmental conditions. Twenty-one, or 53 per cent, of such left rudiments were capable of differentiating into left ovaries which closely resemble the normal left ovary of corresponding age. This capacity for self-differentiation was expressed even though the left gonad rudiment was associated with testicular tissue and grown on a male host. Nineteen, or 47 per cent, differentiated into left ovaries which show a marked reduction in size involving both medullary and cortical components, but especially the latter. In these cases the germinal epithelium becomes flattened, and cortical cords may be few or entirely lacking.

Statistical examination of the data points to a positive correlation between the occurrence of atypical left ovaries and their residence on male hosts. If such is the case, the sex of the host can be considered

as only one contributing factor in influencing the course of differentiation of the engrafted rudiment. This conclusion is based on several facts: (1) Left ovaries, quite normal in appearance, occur in grafts which also contain older testicular tissue and which develop on male hosts. (2) A modified left ovary may arise from a left gonad rudiment associated with left ovarian tissue and grown on a female host. (3) Left ovaries possessing a flattened epithelial covering and showing a marked deficiency in cortical elements arise from left gonad rudiments transplanted alone to female hosts.

Sixteen gonad rudiments (eleven left and five right) gave rise in combination grafts to gonad-like bodies whose sex cannot be definitely ascertained. Since the originally determined sex of the gonad rudiment is known in only five of these cases the data are inconclusive on the question of a possible inhibition of the rudiment's self-differentiating capacity due to hormones secreted by the associated gonad or by the host. The following evidence strongly indicates that these gonad-like bodies do not owe their origin to gonad rudiments developing under the influence of antagonistic sex hormones: (1) Indifferent gonad rudiments which have a male-determining constitution give rise to gonads of unspecific sex, although subject to hormones from associated testicular tissue and from a male host. (2) Similar gonad-like structures whose sex cannot be definitely ascertained arise from gonad rudiments transplanted alone and grown on either male or female hosts.

The originally determined sex of the left gonad which possesses structural characteristics of both a testis and an ovary (Plate II, Fig. 8) was not definitely ascertained, because of the loss of its control partner. It is highly probable, however, that the rudiment possessed a male-determining constitution and that the germinal epithelium persisted for several days after male sex cords had differentiated. Such a persistence of the germinal epithelium on the surface of the left testis of a chick embryo of 12 days' incubation was reported by Laulanié (1886), and the left testis of a late duck embryo described by Burwell (1931) exhibited a similar condition. Persistence of the germinal epithelium on the surface of the right testis has not been reported.

Willier, Gallagher, and Koch (1935) have experimentally stimulated the proliferation of cortex on the surface of the left testis of the chick by injecting theelin, theelol, or male hormone (urine derivative) into the albumin of the incubating egg. The right testis, on the contrary, does not respond to the hormones injected. These investigators attribute the difference in response between the two testes to the presence of a germinal epithelium which develops only on the left testis.

Since the left gonad shown in Plate II, Figure 8, possesses a germinal epithelium but no cortical cords, its origin can perhaps be more correctly attributed to the normal persistence of the germinal epithelium than to a response to hormones.

Although, based on statistical evidence, a positive correlation exists between the occurrence of atypical left ovaries and their residence on male hosts, we are faced with the fact that similar modified left ovaries arise in grafts which are grown on female hosts. It seems necessary to assume, therefore, that some other factor may strongly contribute to this disturbance of the normal course of differentiation which results in modified left ovaries and in the occurrence of gonad-like bodies whose sex cannot be ascertained. This factor may be purely a mechanical one.

Buyse (1935) finds that indifferent gonads of the rat embryo, when transplanted to a subcapsular position in the kidney of an adult male rat, give rise to: (1) testes essentially normal in appearance, (2) ovaries approximating normal structure, (3) ovaries which may show a marked deficiency in cortical elements, (4) ovotestes, and (5) gonads whose sex cannot be ascertained. That this modification is not a "free-martin" effect was proved when similarly modified ovaries were recovered which had arisen from undifferentiated gonad rudiments transplanted to female hosts. Buyse concludes that the modification is probably due to a high susceptibility of the cortical tissue to unfavorable mechanical factors in the graft.

The cortical reduction exhibited by the modified left female gonads in these experiments may result from unfavorable developmental mechanics operating in the graft to which the cortex is more susceptible than is the medullary tissue. In case the mechanical factors are sufficiently unfavorable, normal growth and differentiation

may be inhibited to such an extent that lack of structural organization makes identification of the sex of the gland impossible.

With the exception of a possible influence exerted on the differentiating left female rudiment by its male host, the lack of specific response to hormones on the part of the gonads in the various types of graft and host-graft combinations is striking. Gonad rudiments originally determined male and right rudiments originally determined female, when transplanted in juxtaposition with testes and ovaries from embryonic chicks and grown on either male or female hosts, evidently may differentiate according to their original plan of organization, irrespective of hormones to which they are subjected. Since 28.5 per cent of the left ovaries having a typical structure arose in combination grafts which developed on male hosts, a specific response of the left female rudiment to hormones secreted by the male host may even be questioned.

Ovarian and testicular tissue, known to be producing hormone at the time of transplantation, show no greater capacity for influencing the direction of differentiation of the gonad rudiment in the same graft than do embryonic ovaries and testes. The fact is thoroughly appreciated that no proof exists that the older gonad tissue continues to secrete hormone when transplanted to the membrane. Nevertheless, it is necessary to point out that, in case hormone secretion continues, the hormone is either produced in too small a quantity or does not have the capacity to influence the direction of sex differentiation in the associated gonad rudiment.

Ovaries and testes differentiated as to sex at the time of implantation develop in close proximity or in contact in heterosexual combination for a period of 8 or 9 days and continue to develop in essentially a normal manner.

These facts are interesting when viewed in the light of modifications which occur in the gonads of heterosexual pairs of certain parabiotic amphibia, owing, according to Witschi (1931 and 1934), to an antagonism between cortical and medullary elements which are in close proximity. The data would seem to justify the conclusion that in the chick no such direct local effect is exerted by one gonad upon another of opposite sex as the result of close association in space over a 9-day growth period.

The lack of response on the part of the reproductive organs of the host embryo to the various types of gonad grafts developing on the chorioallantoic membrane corroborates the findings of Willier (1925 and 1927), Kemp (1925 and 1927), Willier and Yuh (1928), and others. Even "multiple-testis" grafts interposed in the blood circulation of the host embryo failed to modify the structure of its reproductive glands and ducts. This does not necessarily imply that these organs are not capable of a response to large quantities of male hormone, but that the dosage necessary for such a response is greater than that which may be supplied by transplantation of the living testicular tissue to the membrane.


SUMMARY

1. Morphologically undifferentiated chick gonad rudiments and older ovarian or testicular tissue were transplanted together on the chorioallantoic membrane of 8- or 9-day embryos, to determine what influence the sex of the associated gonad or of that of the host might have upon the rudiment's capacity to self-differentiate. To ascertain the originally determined sex of the gonad rudiment in the combination graft, its partner gonad from the same donor embryo was transplanted alone to a second host.

2. Right and left rudiments originally determined male and right rudiments having a female-determining constitution were found to possess the capacity to self-differentiate into testes and right ovaries, respectively, regardless of the sex of the associated gonad and of that of the host.

3. Left rudiments, originally determined female, gave rise in such grafts to left ovaries, 53 per cent of which correspond closely to the normal. The remaining 47 per cent exhibit structural modifications involving principally the cortical elements. Although a modified left ovary may develop in combination with left ovarian tissue on a female host and with testicular tissue on a male host, statistical evidence points to a positive correlation between atypical left ovaries and their residence on a male host.

4. Five right and eleven left gonad rudiments in combination grafts gave rise to gonad-like bodies of unspecific sex. These were associated with either ovarian or testicular tissue and developed on

both male and female hosts. Similar structures arise from gonad rudiments transplanted alone to either male or female hosts.

5. Some factor other than the sex of the associated gonad or the sex of the host evidently contributes to the occurrence of the modified left ovaries and the gonad-like bodies whose sex cannot be ascertained. This factor is probably unfavorable developmental mechanics operating in the graft.

6. No evidence was found of a local effect of cortical and testicular (or medullary equivalent) elements upon each other. This is true for combinations of differentiated gonad tissue of opposite sex, as well as for differentiated and undifferentiated gonad combinations.

7. Gonad tissue known to be producing hormone at the time of transplantation is no more effective in altering the course of differentiation of the gonad rudiment in the same graft than is gonad tissue from embryos.

8. Although slight variations in structure occur in the reproductive organs of the host embryos, they are all within the range found characteristic of the normal embryo.

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# CATALASE ACTIVITY DURING EMBRYONIC DEVELOPMENT (ACRIDIDAE, ORTHOPTERA)[1]

(Three figures)

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SEVERAL theories exist as to the possible functions of the enzyme catalase. The earliest of these considered its activity to be directly correlated with respiration and general metabolism (Alvarez and Starkweather, 1918; Appleman, 1916, 1918; Burge, 1922, 1926; Burge and Leichsenring, 1922; Davis, 1930; Gustafson, Clark, Shaw, and Warweg, 1932; Neller, 1931). Other investigators have found no indication of such a correlation (Alexeeff and Russinowa, 1931; Bialascewicz, 1921; Bodine, 1921; Crocker and Harrington, 1918; Doyle and Clinch, 1928; Morgulis, 1921; Pope, 1933; Rhine, 1924; Stehle, 1919; Stern, 1927). More recently Dixon (1925) has shown that the enzyme acts as an antitoxin by decomposing hydrogen peroxide produced in certain cellular oxidations. Callow (1923) marks a similar observation based on differences in catalase activity of aërobic and anaërobic organisms (Kluyver, 1924; Morinaga, 1925; Stern, 1927). Lantz (1927) thinks the function of the enzyme may be to prevent excessive oxidation, while Staffe (1931) suggests that it may have an oxygen-sparing action.

Several workers have investigated catalase and its relation to cellular activity during the course of embryonic development of insects (Fink, 1930; Spooner, 1927; Steche and Waentig, 1912; Zeiger, 1915). However, since little definite information is available about this subject, a detailed study of changes in catalase activity during the entire embryonic development of *Melanoplus differentialis* has been made. The egg of this grasshopper has proved to be particularly suitable for such an investigation. This egg, in its normal course of development, passes through a period of marked cellular activity, followed by a resting or diapause stage, after which

[1] Aided by a grant from the Rockefeller Foundation for work on cellular physiology.

active development continues up to the time of hatching. The diapause, or developmental block, may be broken experimentally by exposing the egg to a low temperature, such as 5° C., for an appropriate period of time. Since rates of oxygen intake have been established and correlated with the various developmental activities of this egg (Bodine, 1929; Bodell, 1935), it seemed desirable to investigate the development of, and the possible rôle played by, the egg catalase.

Catalase activity has been measured by the oxygen produced from the decomposition of hydrogen peroxide. The volume of this gas was determined by means of the especially adapted mercury manometer shown in Figure 1. The manometer vessel (*A*) is equipped with a side chamber (*B*), making it possible to keep extract and hydrogen peroxide separate within the vessel. Paired openings (*C* and *D*), one in the neck of the flask and one in the base of the manometer, may be superimposed, thus leaving the vessel open to the air during temperature equilibration. By rotating the vessel, the system is closed off from the outside.

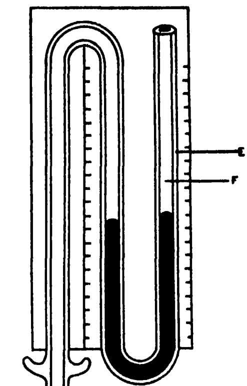


FIG. 1.—Type of manometer used in experiments. Volume of vessel (*A*), 25 cc.; volume of side chamber (*B*), 0.8 cc.; length of tube (*E*), 220 mm.; diameter of tube (*F*), 2 mm.

In all experiments, twelve manometers were placed on a mechanical shaker, in a water bath kept at 25° ±0.2° C. At the end of a 10-minute equilibration period, they were closed and set in motion, thus thoroughly mixing the contents of the flasks. In all cases the end-point of the reaction was reached within an hour, and this length of time was considered a minimum for the duration of experiments.

Eggs used were of three types: (1) those kept constantly at 25° C., on moist filter paper; (2) those exposed to low temperatures (5° C.) during the pre-diapause period and later placed at 25° C.; and (3) those exposed to 5° C. during diapause and subsequently kept at 25° C.

The average developmental stage of any series of pre-diapause eggs was determined by removing the embryos from a ten-egg sample. Morphological stages of the embryo in diapause and post-diapause eggs were determined by noting eye position or by removal of the chorion overlying the transparent cuticle (Slifer, 1932). Chorion removal has no effect on catalase activity, as eggs used with or without it gave similar results.

Since embryos in diapause and those in early post-diapause are morphologically similar, the physiological states of such eggs were determined by measuring respiratory rates in a differential manometer (Bodine, 1929).

Enzyme extracts were prepared by grinding one or more eggs with 2.5 cc. of distilled water in a glass mortar. Eggs ground with or without sand gave similar results. An 0.8-cc. portion of the extract was used in each determination, and catalase activity has been expressed as cubic centimeters of oxygen per egg.

Hydrogen peroxide solutions were made up daily and adjusted with phosphate buffer to a pH of 6.8–7.0, which for general purposes may be considered an optimum hydrogen-ion concentration range for the catalase reaction (Morgulis, 1921). The buffered peroxide was kept in colored glass bottles, and standardized daily with N/0.1 $KM_nO_4$. Figure 2 shows graphically the relation between the amount of oxygen evolved and the concentration of hydrogen peroxide when the same egg extract was allowed to react with varying hydrogen peroxide concentrations. From an inspection of this figure, it will be noted that the maximum gas evolution occurs when N/0.24–0.40 $H_2O_2$ is used.

In each experiment, a blank manometer containing only hydrogen peroxide and distilled water or boiled extract was prepared in order to correct results for possible changes in atmospheric pressure and spontaneous decomposition of peroxide. If sand had been used in the preparation of test extracts, it was also included in the control manometer.

Oxygen volume was calculated from manometric readings as follows: $(R_1 - R_0)K = V$, where $V$ equals volume of oxygen in cubic centimeters; $R_1$ and $R_0$, total millimeter change in the readings of test and control manometers, respectively; and $K$, the constant of

the manometer for oxygen at standard conditions of temperature and pressure.

The amount of variation in total oxygen volume due to individual differences in manometers, and perhaps to irregularities of technique, has been repeatedly determined by using uniform extract and hydrogen peroxide in a series of eleven manometers. The maximum variation in gas volume in any one experiment did not exceed 0.0011 cc.

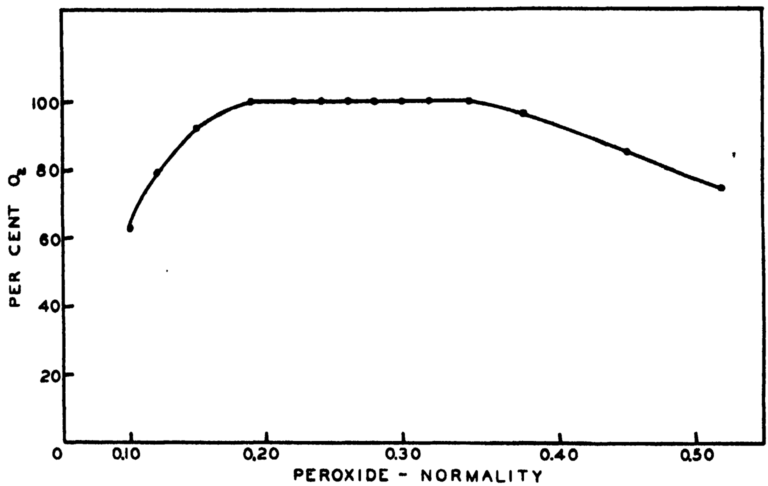


FIG. 2.—Catalase activity with variations in $H_2O_2$ concentration (100 per cent equals optimum oxygen production).

## RESULTS

The relative catalase activity and respiration of the eggs is shown graphically in Figure 3. Each point indicated represents the average value for a group of at least fifty eggs. It will be noted that from the time of laying, through the eighth day of development, there is practically no change in catalase activity. During this period, ten eggs were used in the preparation of each extract in order to obtain significant readings. In all subsequent stages, however, individual eggs were employed. From the ninth to the twenty-first day, or through pre-diapause, a marked increase in catalase activity occurs. During early diapause (23–30 days) it is unchanged, but from the thirtieth to the fortieth day it increases rapidly. Statistical compari-

son of data covering the 28–32 and the 39–41 day stages indicates significant differences in catalase activity between these two periods. Maximum catalase activity reached on the forty-first day continues throughout diapause. All post-diapause determinations have been made on eggs which were kept at 25° C. after they had been exposed

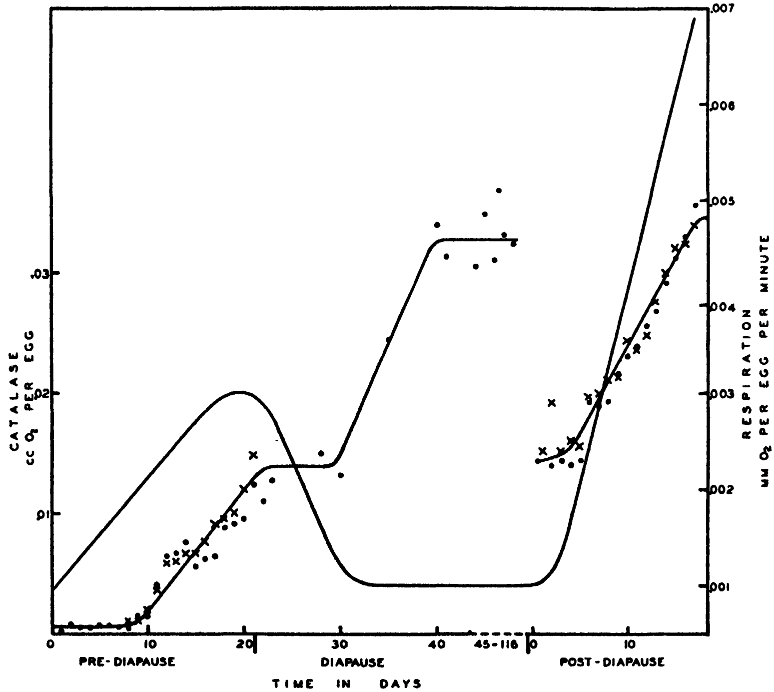


FIG. 3.—Catalase activity and respiratory rate of eggs from laying to hatching. Ordinates: at left, cubic centimeters of $O_2$ evolved from $H_2O_2$ by catalase per egg; at right, relative $O_2$ intake per egg per minute. Abscissa, days of development. Dots, eggs exposed to 5° C. during diapause. Crosses, eggs exposed to 5° C. during pre-diapause. Respiratory curve (without dots or crosses) adapted from Bodine (1929).

to cold (5° C.) for several months in order to remove the diapause block. Catalase activity during the first 5 days of post-diapause remains at the level which is characteristic of early diapaues (23–30 days). From the sixth day of post-diapause until hatching, the amount of catalase in the egg increases rapidly to the maximum. There was no change shown in catalase activity at the time of hatching or during the first instar.

It will be noted that throughout developmental periods there is no difference shown between the catalase activity of eggs which had been exposed to cold during pre-diapause and that of eggs not exposed to cold until in the diapause stage.

An attempt has been made to determine the relative distribution of catalase within the egg. Separation of yolk from embryo is easily accomplished in all stages of development. After yolk engulfment, the yolk may be freed from the embryo by puncturing the posterior

TABLE I

DISTRIBUTION OF CATALASE ACTIVITY BETWEEN (1) EMBRYO AND (2) YOLK PLUS EXTRA-EMBRYONIC EGG MEMBRANES AND FLUIDS

(Each figure represents average result from twenty eggs)

| Embryonic State | Percentage of Catalase Activity | |
|---|---|---|
| | Embryo | Yolk Extra-embryonic Membranes and Fluids |
| 10 days | 5 | 95 |
| 15 days | 5 | 95 |
| Diapause (50 days) | 5 | 95 |
| Post-diapause (15 days) | 65 | 35 |

end of the abdominal cavity and forcing the yolk out through the aperture. As is indicated in Table I, during pre-diapause and diapause the yolk, plus egg membranes and fluids, contains 95 per cent of the total catalase activity, only 5 per cent being found in the embryo. However, after yolk engulfment, or in late post-diapause, the embryo (measured 2 days before hatching) contains 65 per cent of the enzyme, and the yolk 35 per cent. Only in rare cases was catalase activity observed in extracted shells. It was assumed that such activity in shells was due to incomplete removal of the yolk and fluids. It seems, therefore, that most of the catalase is concentrated in the various extra-embryonic fluids and cellular structures until after yolk engulfment, when the situation is reversed.

If one compares relative catalase activity with changes in the respiratory rates of similar eggs (Bodine, 1929; Boell, 1935), it is evident, as shown in Figure 3, that no correlations seem to exist.

As no mitotic spindles are found during diapause (Slifer, 1931) and as catalase activity is high during this period, there seems to be no apparent or significant correlation between cell activity and catalase.


SUMMARY

1. A quantitative study has been made of catalase activity in the egg of the grasshopper, *Melanoplus differentialis*, throughout its entire period of development.

2. Catalase activity is at a minimum for 8 days after laying. It increases from the ninth to the twenty-first day, is fairly constant from the twenty-first to the thirtieth day, and rises (30–40 days) to reach a maximum (40 days) retained throughout the remainder of diapause. After diapause, in eggs which have been cold-treated, catalase is at the 21–30-day level for a period of 5 days. From the sixth day of post-diapause until hatching, the enzyme increases to a maximum.

3. Exposure to low temperature (5° C.) has no effect on the subsequent catalase activity (at 25° C.) of pre-diapause eggs.

4. Before yolk engulfment, 95 per cent of catalase activity is found in the yolk, egg membranes, and fluids, and 5 per cent in the embryo; after engulfment, 65 per cent in the embryo, and 35 per cent in the yolk, extra-embryonic structures, and fluids.

5. No correlation seems to exist between respiratory rate and catalase concentration or activity.


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# RECIPROCAL INHIBITION AND ITS REVERSAL BY STRYCHNINE IN THE MODIFIED CTENOPHORE, COELOPLANA BOCKII[1]

(Two figures)

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OF FIRST importance in maintaining the integrity of muscular movement in the individual are two properties of the nervous system, namely, rapid conduction of impulses and reciprocal inhibition. The first is apparent wherever nervous tissue is found; the second occurs where muscles are paired so that if both contract at the same time they act antagonistically and nullify movement. Reciprocal inhibition apparently is absent in coelenterates but occurs in echinoderms and worms. It therefore becomes a nice question to determine to what degree anatomical complexity of the nervous system is necessary for this function. In the vertebrate, where the problem has been most studied, the arrangements for reciprocal inhibition in voluntary movements are exclusively central. On the other hand, the muscles which open and close the pincers of the great claw of the crayfish show reciprocal inhibition even when the appendage is detached; hence the mechanism in this case is peripheral. It is also of interest to note that the intestine of the vertebrate, in which the musculature is innervated by a nerve-net plexus, shows reciprocal inhibition in the involuntary movements of peristalsis. Therefore, the plexus must be adequate to the reaction in this highly special case. In fact, Magnus and Wolf (1913), as a result of their work on the reflexes of the skeletal musculature of the mammal, concluded that reciprocal inhibition cannot be explained on the basis of any certain anatomical scheme of nervous

[1] It gives me pleasure to express my heartiest thanks to Mr. M. Eri and Mr. Y. Yoshii for many courtesies extended to me during my stay at Misaki, and to Professor Komai for helpful advice on the structure of *Coeloplana*.

[2] Visiting professor of biology of the Rockefeller Foundation at Tohoku Imperial University, Sendai, Japan, 1933–34.

connections. Reciprocal inhibition, therefore, must depend upon a special functioning of a sensory-motor neuron pattern, which may vary in its form in representatives of the different phyla.

Echinoderms, worms, mollusks, and crustaceans all show reciprocal inhibition. In coelenterates the nerve-net system apparently functions exclusively in conducting and shows inhibition only when excitation is applied so that two impulses arrive at an effector from opposite directions (Moore, 1926). The evidence goes to show that ctenophores behave in the same way. I found it, therefore, of considerable interest to study the reactions of a creeping form of the modified ctenophore, *Coeloplana bockii* (Moore, 1933), with a view to discovering whether, in a form structurally so elementary, reciprocal inhibition plays a part in locomotion.

The nervous system of *Coeloplana* is, according to Komai (1922), of the simplest net type, without any evidence of concentration into ganglia or strands. Yet, reciprocal inhibition can be demonstrated in this form. The adult *Coeloplana* is a flat, slowly creeping animal which superficially resembles a flatworm. It is found living symbiotically on colonies of *Alcyonaria* in the shore waters of Indo-China and southern Japan. The free-swimming larva is, in appearance, a typical ctenophore with the characteristic eight rows of paddle plates. The body of the adult is formed from the embryo by the outward growth of the pharynx, a process which begins as soon as the young animal settles down with its oral opening in contact with a surface. As this development proceeds, the paddle plates disappear and only the two branched tentacles remain to bear witness to the animal's ctenophore history. The tentacles determine the long axis and the bilateral symmetry of the animal; but in creeping, it may move equally well in any direction—sidewise, forward, or backward.

The experiments to be described were carried out on a large number of individuals in June and again in November at the Misaki Station. The results were uniformly the same in all cases, and the facts described are therefore well established.

If an active *Coeloplana* is touched at any point on its surface—say the lateral margin—that region moves away by withdrawing centralward as a result of the contraction of the transverse musculature, while the opposite side extends by virtue of the relaxation of its

transverse musculature, the total effect of both movements being to carry the animal away from the point of excitation (Fig. 1). This constitutes a simple case of reciprocal inhibition as regards the transverse musculature of the contralateral side. The pattern of the response is changed, however, if the animal is kept for a few minutes in a solution of strychnine sulfate in sea water, 1 : 50,000.[3] If it is now stimulated on the lateral margin, as in the first case, there is the usual

FIG. 1

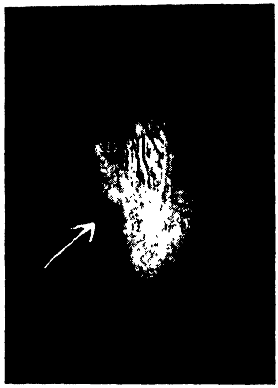

FIG. 2

FIG. 1.—Normal specimen of *Coeloplana* responding to mechanical stimulation applied at the locus indicated by the arrow. The result: withdrawal toward center by the ipsilateral side and movement away from the center by the contralateral side.

FIG. 2. Strychninized specimen of *Coeloplana* responding to mechanical stimulation as in Figure 1. The result: withdrawal toward center by both sides, showing reversal of response under strychnine for the contralateral side.

withdrawal from the locus of excitation toward the center; but the opposite side also reacts in an identical fashion by moving centralward, which actually carries that side *toward* the locus of excitation (Fig. 2). This is the result of the contraction of the contralateral transverse muscles which, in the normal response, are inhibited. Strychninization has thus changed the reciprocal inhibition of the muscles of the contralateral side into an excitation—a conversion which causes *Coeloplana* to react to stimulation from a single point just as a medusa does, by a bending of the body toward that spot.

[3] Atropine sulfate, caffeine, nicotine, and phenol in dilute solutions in sea water cause hyperexcitability and spasmodic contractions of the musculature in *Coeloplana*, effects similar to those produced by strychnine. Only atropine, however, yields reversal effects comparable to those of strychnine.

Specimens of *Coeloplana* in which a central disk, including the "aboral sense organ," had been excised, reacted in every way like an intact animal. From this it is concluded that the nerve net of the body is adequate to mediate reciprocal inhibition and strychnine reversal. *Coeloplana* thus furnishes an instance of a form, without morphological evidence of central tracts or ganglia, which possesses neurons which are functionally and chemically developed so that they react in a typical way to bring about reciprocal inhibition and its reversal under strychnine. This is, however, not a common property of sensory and motor neurons, for sea anemones have both; but I have never been able to observe any excitatory effects of strychnine on the body musculature of these animals, even when they were kept for a time in a concentrated solution of strychnine in sea water.

The experiments with strychnine are interesting, as they throw some light on the question of the extent to which strychnine reversal of reciprocal inhibition takes place. In recent years a number of exceptions have been found to the original rule of Sherrington (1898), according to which the inhibition phase of reciprocal inhibition is transformed into an excitation by strychnine. Dusser de Barenne (1933) lists the exceptions to the rule. Thus, the tonic neck-reflexes, the compensatory eye-reflexes, and the caloric, labyrinthine eye-reflexes are unaffected by strychnine even in doses large enough to produce convulsions. Although strychnine reversal does not occur in these reflexes, the same muscles show typical reversal when activated in other reflexes. Similarly, inhibition of the extensor muscles in decerebrate rigidity, which is elicited by stimulation of the anterior parts of the cerebellar cortex, and inhibition of the opening muscles of the mouth in the mandibular and linguo-mandibular reflex are not reversed by strychnine. For these reasons Dusser de Barenne suggests that reciprocal inhibition which can be reversed by strychnine represents a special case in which "the inhibitory stimulus applied to the afferent peripheral nerve includes also an excitatory factor, which normally does not predominate, but under strychnine becomes dominant and blocks the inhibitory effect, whereas the absence of this reversal is probably due to the pure inhibitory nature of the excitation." It should be borne in mind that the exceptions to strychnine reversal occur in the vertebrates, chiefly mammals, and, so far as we

know, are confined to that phylum. The fact that reciprocal inhibition and its reversal by strychnine are of such general occurrence among the invertebrates, it seems reasonable to think, puts the matter in a somewhat different light. Since reciprocal inhibition can be shown to occur in *Coeloplana*, starfishes, flatworms (Moore, 1918), and annelids (Knowlton and Moore, 1917), we have sufficient evidence of its widespread distribution in the most diverse morphological types of nervous system. That strychnine reversal is the rule in all these cases indicates the fundamental nature of the reaction. It is suggestive and possibly significant that in the mammal the "recruitment" reflexes, which resemble those of the invertebrates in their long latency, slow onset, long after-discharge and slow decline, are profoundly affected by strychnine (Bremer and Rylant, 1926). It would seem, therefore, that the mechanism for pure inhibition which is not susceptible of strychnine reversal is a later, special development in the vertebrate.

The results with *Coeloplana* furnish an instance of extreme simplicity of neuronic structure which is, nevertheless, adequate to function as typical reciprocal innervation in locomotor movements. This gives support to the position of Magnus and Wolf according to which reciprocal inhibition depends for its mediation upon physiological, rather than special anatomical means. It may be regarded as established that for the mediation of reciprocal inhibition, sensory and motor neurons linked together in very simple histological pattern are adequate, and that in their chemical affinities, as indicated by the results of strychninization, they are equal to neurons of similar function in more complicated nervous systems.

SUMMARY

1. Specimens of *Coeloplana bockii*, which possess only a nerve-net system, show reciprocal inhibition of the transverse musculature as a result of contralateral excitation.

2. In strychninized specimens reciprocal inhibition is reversed and excitation results from contralateral stimulation.

3. Individuals from which a central disk, including the "aboral sense organ" had been removed, reacted similarly to the intact animals. Hence the neural reactions are mediated by the diffuse system of the body.

4. The results of the experiments support the view of Magnus and Wolf to the effect that reciprocal inhibition depends for its mediation upon physiological means rather than upon special anatomical arrangements.

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# KILLING ORGANISMS WITH CHROMIUM AS FROM INCOMPLETELY WASHED BICHROMATE-SULFURIC-ACID CLEANED GLASSWARE

(One figure)

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POTASSIUM BICHROMATE is an important component of many of the killing and fixing fluids of the biologist, as well as of the bichromate–sulfuric-acid mixture often used for cleaning glassware. Because the bichromate adsorbs to glassware and is washed out with difficulty, it is necessary to investigate the dangér involved when this mixture is used for cleaning glassware and other articles to be used with living organisms. Now that the amount of chromium which may remain after a certain amount of washing is known, the effect of this amount of chromium on certain test organisms is here reported.

## I

Laug (1934) has found that S-diphenylcarbohydrazide may be used to determine colorimetrically as little as 0.1 μg.[1] of potassium bichromate per milliliter of solution. He placed sulfuric-dichromate cleaning fluid in different kinds of glassware for varying lengths of time. The dishes were then washed in seven changes of tap water and rinsed with three different lots of distilled water. After this washing, Laug proceeded to extract much of the remaining bichromate with different amounts of water remaining in contact for different lengths of time, and was able to recover, on the average, 0.025 μg. of bichromate per milliliter of wash water. When smaller amounts of water were used in the dishes, as in an experiment with living organisms, the bichromate concentration might become as high as 1.0 μg./ml. even after ten washings of the glassware. This makes it necessary to determine whether amounts of from 0.1 to 1 μg. of bichromate per milliliter of solution are toxic to living organisms.

[1] A microgram (μg.) or gamma (γ) is $10^{-6}$ gram.

## II

The first test organism used was the yeast *Saccharomyces cerevisiae* Hansen because of its convenience and the standard conditions known for yeast growth (Richards, 1932, 1934). In each series part of the tubes of Williams' culture medium were retained as controls, and to the other tubes various known amounts of potassium bichromate were added.[2] The results from three separate series of experiments are summarized in Figure 1. The ordinate values give the percentage of yeast in the tubes of a given bichromate concentration of that of the control tubes as measured with a photoelectric nephelometer (Richards and Jahn, 1933). With very dilute solutions of potassium bichromate (0.0025 to 0.04 μg./ml.) the bichromate adsorbs to the glass and gradually redissolves into the culture fluid, and the bichromate shows greatest toxicity about 60 hours after the populations were seeded. As the cells are killed, the bichromate seems to be bound by the killed cells; and the resulting decreased concentration of the bichromate permits nearly complete recovery of the population of yeast. With stronger concentrations the amount of killing is greater at first; and as the bichromate is removed by the killed yeast, there is a corresponding recovery which is less complete as the concentration of the bichromate increases. A concentration of about 10 μg./ml. of bichromate sterilizes the culture.

It was shown (I) that the amounts of bichromate that might be found after ten washings of the glassware were from 0.1 to 1 μg/ml. These concentrations reduce the yeast populations from 22 to 47 per cent within 24 hours after seeding, and the ultimate recovery is not complete. The reliability of yeast measurement is such that deviations of 4 or more per cent indicate real differences beyond any normal variation. Consequently, concentrations as low as 0.0025 μg./ml. would invalidate experiments made with yeast.

## III

*Spirogyra insignis* Hess and *S. tenuissima* Hess placed in dilute Knop's solution appeared normal and grew well for the 15 days' duration of the experiment (Table I). When the test amounts of 0.1 to 1 μg./ml. bichromate are added to similar amounts of Knop's

[2] For the effects of massive doses of $Cr_2(SO_4)_3$ on yeast, cf. Hébert (1907).

solution, one-quarter of the filaments placed in the test solutions are injured or killed within a day. These injured filaments bind and

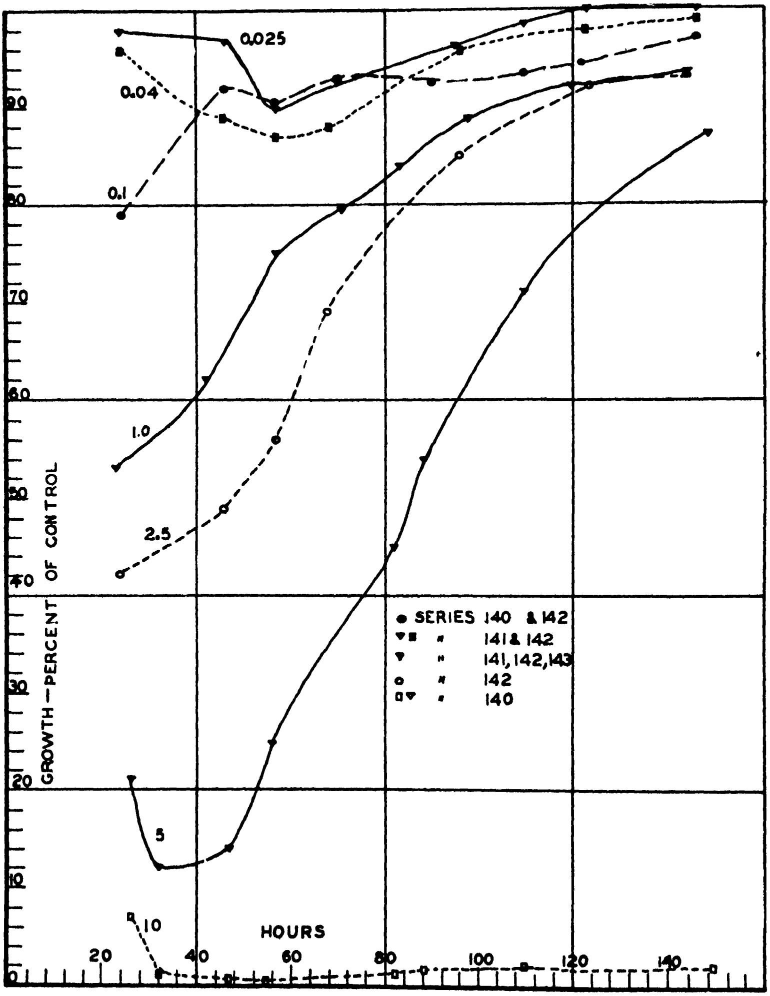


FIG. 1

reduce the poison; and later, with the lesser concentration, the plants nearly recover to the same condition as in the controls; but with the greater concentration only half of the algae recover. Fewer filaments of the smaller species recover than of the larger

species. In neither case was more than fair preservation accomplished by 100 μg./ml. While greater concentrations were not tested, it would appear that less bichromate than usually found in killing

TABLE I

| | Control | 0.1 μg. | 1 μg. | 5 μg. | 10 μg. | 50 μg. | 100 μg. |
|---|---|---|---|---|---|---|---|
| *Spirogyra insignis:* 23 hours.. | Normal and growing | 25 per cent killed and blackened | 25 per cent killed | Dead and slightly plasmolyzed | Plasmolyzed | Plasmolyzed | Killed and fixed |
| 48 hours.. | Same | Same | 35 per cent killed | 50 per cent killed | Same | Same | Same |
| 120 hours. | Same | 15 per cent dead | 50 per cent killed | 95 per cent killed | Plasmolyzed | Same | Fair preservation |
| 15 days... | Same | 90 per cent recovered | 50 per cent normal | All dead | 10 per cent preserved | 20 per cent preserved | Fair preservation |
| *Spirogyra tenuissima* | Normal and growing | This species is more sensitive to the poison and is less well preserved in the higher concentrations. Only half of the filaments recovered in 0.1 μg./ml. in 15 days | | | | | |
| *Oscillatoria limosa:* 23 hours.. | Actively motile | Motile | Motile | Motility reduced | No motility | Killed | Killed and fixed |
| 120 hours. | Actively motile and growing | Few moving cells | 80 per cent dead and plasmolyzing | All dead and plasmolyzing | All dead and plasmolyzing | Few preserved | 70 per cent preserved |
| 15 days... | Same | 50 per cent motile | 25 per cent motile | 5 per cent motile | All disintegrated | Fair preservation | |
| *Raphidium* sp.: 120 hours. | Active | Less active | Few motile | 4 per cent dead | 50 per cent dead and disintegrating | 99 per cent dead; fair preservation | |
| 15 days... | 0 per cent dead; active motility | 12 per cent dead | 5 per cent dead | 25 per cent dead | 67 per cent dead | 80 per cent dead | 90 per cent dead |
| | | Motility greatly reduced with those still living | | | | Fair preservation of dead | |
| *Chlorococcus* sp.... .. | Up to 5 μg./ml. little effect was noticed. Greater concentrations kill and none survived the higher concentration; and preservation was poor | | | | | | |

and fixing solutions might be used to advantage for the preservation of algae.

The blue-green alga *Oscillatoria limosa* Agardhi was tested in the same manner as *Spirogyra;* and while the injury was somewhat less during the first day, the later injury to the filaments was greater and

less recovery took place than with *Spirogyra* at the end of 15 days (Table I). Both of these algae are severely injured in amounts of bichromate that might be found when only ten washings were used following the use of sulfuric-bichromate cleaning fluid.

The diatoms and desmids tested (Table I) are less injured by such small amounts of potassium bichromate than the blue-green and green algae. With the diatom (*Raphidium*), the upper possible limit (1 μg./ml.) may produce sufficient injury or killing to vitiate the use of it as a test organism in incompletely washed bichromate–sulfuric-acid-cleaned glassware.

## IV

The danger of bichromate poisoning from inadequately cleaned glassware is less when the culture fluids are alkaline, because less bichromate will remain in solution; and this is true also for sea water. Ten micrograms per milliliter and greater concentrations in sea water were found to be toxic for the developing sea-urchin egg (*Arbacea punctulata* Lamarck).[3] Until the toxicity of bichromate to other marine organisms is known, care should be taken to avoid the possibility of bichromate contamination from inadequate washing of vessels treated with bichromate-sulfuric-acid solution.

## V

The effect of potassium bichromate on the developing *Amblystoma punctatum* egg was determined by placing the eggs in tap water containing known amounts of the chemical and comparing the development with controls in the tap water without the chemical. A very small amount (0.0001 μg./ml.) delayed the development at first by about two Harrison stages, and a few days later the animals appeared to be four stages less developed than the control animals. The larvae hatched a day or two earlier than the control animals because the capsule was weakened by the bichromate. About half of the larvae developed a kyphotic bending of the body shortly after hatching; and their swimming movements were irregular, leading to circus movements, or to tetanic paralysis on slight stimulation. The total mortality was about 16 per cent, while none of the control animals were dead when the experiments were terminated within a

[3] I am greatly indebted to Dr. Ethel Brown Harvey for making the tests with *Arbacea*.

week after hatching. About half of the animals appeared to be uninjured, but most of these were one stage less developed when they hatched.

With concentrations of 0.001 $\mu$g./ml. and stronger, no normal animals appeared. This concentration retarded development about two stages, and the mortality was 33 per cent. The first evidence of injury appeared within 2 days as an incomplete closure of the neural folds and an extrusion of some yolk material. The injury led to marked deformities during development and to disturbed equilibrium and swimming of the tadpoles on hatching.

The same kind of early injury and deformity occurred when the concentration was 0.01 $\mu$g./ml.; the mortality was 36 per cent; and at the end of the experiment the average length of the larvae was 8 per cent less than that of the controls. A slightly higher concentration, 0.1 $\mu$g./ml., also resulted in early injury and deformity; and many of the animals were dead within a period of 4–10 days. The length of the gills was decreased by about 20 per cent; the total length was 10 per cent less; and the mortality was 55 per cent.

Increasing the concentration to 0.5 $\mu$g./ml. increased the total mortality to 80 per cent; and there was less early injury but greater later injury, which was probably due to a tanning of the mucin capsule retarding the penetration of the bichromate. Considerable reduction of the gills and a marked increase of mucus secretion on the surface of the animal were observed.

One microgram per milliliter, the greatest concentration that might occur when only ten rinsings of glassware were used, showed a definite hardening of the capsule and of the vitelline membrane. In one case the animal reached stage 40 and died without being able to rupture or dissolve the vitelline membrane. In most cases the animal was deformed in the capsule before hatching. Many were kyphotic; and others had frilled, deformed tails. If the animal appeared normal in form at hatching, the swimming movements were impaired and a twisting of the body occurred within 2 days after hatching. All of the animals had dwarfed and deformed gills and a hypersecretion of mucus. The average length of the animals at the end of the experiment was 12 per cent less than that of the controls, as nearly as one could measure around the deformed bodies to esti-

mate their length. The mortality was a little less, 60 per cent, because the tanning of the capsule lowered the early mortality; but none of the survivors were in any way normal.

Greater concentrations of potassium bichromate than 1 $\mu$g./ml. penetrated the hardened capsule still less, so that the early injury was less and the retardation of development averaged only one Harrison stage. Hatching was delayed from 1 to 5 days because of the hardened capsule, and the injury immediately following hatching quickly paralyzed the animals.

The developing salamander eggs are more sensitive to small amounts of bichromate than any of the other organisms used in the experiments. Sulfuric-bichromate cleaning fluid should never be used on glassware intended for the rearing of these animals.

## VI

Can the adsorbed traces of bichromate which may be toxic to living organisms be removed after clearning with the sulfuric-bichromate cleaning fluid? Laug (1934) has demonstrated that the amount recovered in successive portions of wash water is greatest at first and gradually becomes less. Hot water was found to be over three times more efficient than cold water. He recommends successive periods of at least 15 minutes in boiling water as the best method for removal of the bichromate.

Small, nearly closed vessels, such as are commonly used in microrespiration apparatus (Warburg, etc.), are freed from adsorbed cleaning fluid with difficulty; and especial vigilance should be maintained to make certain that the washing is adequate. Improper washing of the vessels may be responsible for some of the erratic and divergent results sometimes obtained with this type of equipment.

Unless it is known that the washing is sufficiently complete or that the organisms used are not affected by the amount of bichromate remaining, it would seem better to avoid the use of this cleaning mixture. Ten per cent nitric acid will remove many of the residues found on glassware, washes out easily, and is volatile in case traces are not washed out. Another good cleaning solution is 1–5 per cent trisodium phosphate. This probably is not very toxic to organisms in amounts that would not wash out readily. The efficacy of many soap compounds is due to the addition of this alkali. The

writer has found that Sapolio, with the vigorous use of a good brush, is quite adequate for cleaning glassware to be used for living organisms, and that it washes off easily. Pipettes, etc., which cannot be reached by a brush may be cleaned by the less toxic compounds.

## SUMMARY

Concentrations of from 0.1 to 1.0 µg./ml. of bichromate may occur when small amounts of water are used in dishes which have been cleaned with potassium-bichromate–sulfuric-acid cleaning fluid and then washed with seven changes of tap water and three of distilled water. Laug has further demonstrated the difficulty of removing these remaining traces of bichromate from glassware. The foregoing amounts of potassium bichromate are shown in this paper to be sufficiently toxic to invalidate experiments made with yeast, *Oscillatoria*, two species of *Spirogyra*, and developing *Amblystoma* eggs. A desmid and a diatom were less sensitive to potassium bichromate, but the former (*Raphidium*) was injured by 1.0 µg./ml. of bichromate. Only 50 per cent of the *Amblystoma* hatched as normal larvae when the bichromate concentration was 0.0001 µg./ml. Unless it is known that the washing of glassware cleaned in sulfuric-acid–bichromate fluid is adequate, or that the organisms used are not poisoned by such traces as may remain, it is recommended that less-toxic cleaning fluids be used. The cleaning of microrespiration apparatus should receive special attention. Other cleaning materials that wash off more easily are Sapolio, 10 per cent nitric acid, and 1–5 per cent trisodium phosphate.

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# STUDIES ON THE PHYSIOLOGY OF AMOEBA I. THE RELATION BETWEEN NUTRIENT SOLUTION, ZONE OF GROWTH, AND DENSITY OF POPULATION

(Three figures)

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THE general culture conditions in a culture of amoebae, free of bacteria, were discussed in a previous study (Reich, 1935). It was observed there that in a semiliquid nutrient solution the amoebae grow in a distinctly formed zone of growth at a depth of 5–7 mm. below the surface. The purpose of the present study was to find out whether the adjustment to a relatively low oxygen pressure is a constant quality and could, as such, characterize the species, or whether it depends upon various external factors. The first experiments with different H-ion concentrations actually showed that the depth of the zone of growth may be affected. It could be seen, at the same time, that, as the density of population increases, the zone of growth descends farther and farther down. The question arose as to whether the depth of the zone of growth is indicative of the oxygen requirement of the amoebae or whether it depends simply on the weight of the amoeba layer, which becomes the heavier and sinks the deeper, the richer it is in individuals. It was necessary, therefore, to look for factors which would affect the number of individuals without changing thereby the depth of the zone of growth.

## EXPERIMENTS WITH VARIOUS H-ION CONCENTRATIONS

In these experiments the nutrient solution previously indicated (Reich, 1935) was used with addition of 0.15 per cent agar. By adding small amounts of NaOH or HCl, the desired pH was obtained. Five cubic centimeters of this solution were poured into each of several test tubes, the diameters of which were equal as far as possible; and then the portions were sterilized. Special care was exercised to use only simultaneously prepared samples of nutrient

solutions in each series, in order to avoid errors which might result from minute differences in the composition of the solutions. The amoebae to be inoculated were taken only from well-growing cultures previously centrifuged and washed five times with 0.1 per cent NaCl solution. Each test tube was inoculated with 0.5 cc. of the amoeba suspension. The first experiment comprised the following H-ion concentrations: pH 8.2, 7.6, 7.0, 6.7, 6.6, 6.2, 6.0, 5.8, 5.7. Figure 1 and Table I illustrate the results of a one-month culture. The figure shows clearly that in an alkaline medium there exist differences in the depths of the zones of growth; whereas in solutions

FIG. 1.—The different depths of the zones of growth in cultures of amoebae of various H-ion concentrations. The numbers indicate the various pH values. The not-inoculated control medium of pH 6.8 is marked with *O*.

of pH 6.7 and less, the zones appear roughly at the same depth. A second characteristic feature to be mentioned is the fact that, while at pH 8.2 and pH 7.6 the downward distribution of the amoebae in the solution is uniform and an indistinct boundary appears only toward the top, from pH 7.0 on, the zone of growth is markedly limited, both below and above. The reason for the impression of a more pronounced upward boundary is probably that the downward-wandering amoebae drag the agar particles along with them, thereby effecting a clarification of the upper strata of the semiliquid medium. The test tube marked with an *O* in the figure serves as a control containing a not-inoculated nutrient solution of pH 6.8. It shows that this process of stratification is not a pure precipitation phenomenon of the agar colloidal particles but that it is brought about by the growth of the amoebae.

In order to determine the number of amoebae present, the cultures were well shaken and 10 drops of each culture were counted, drop by drop, in a Thoma counting-chamber. From each group two test tubes were selected, so that the numbers in the table represent the average values for every twenty counts and refer to 0.1 cu. mm. of solution.

In Table I are indicated the number of amoebae, the number of cysts, and the sum of the two. The table shows that the more acidic the solution the greater is the number of amoebae.

Two days after counting the amoebae, the normal stratification of the cultures reappeared. One month later, i.e., after two months

TABLE I

THE EFFECT OF H-ION CONCENTRATION ON THE DENSITY OF POPULATION IN A ONE-MONTH CULTURE

| | pH | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 8.2 | 7.6 | 7.0 | 6.7 | 6.6 | 6.2 | 6.0 | 5.8 | 5.7 |
| Amoebae........ | 1.2 | 5.1 | 8.4 | 9.8 | 10.9 | 11.9 | 12.5 | 11.2 | 11.8 |
| Cysts............ | 0.2 | 0.6 | 1.4 | 3.0 | 2.2 | 4.7 | 3.3 | 3.8 | 4.2 |
| Total........ | 1.4 | 5.7 | 9.8 | 12.8 | 13.1 | 16.6 | 15.8 | 15.0 | 16.0 |

of culivation, the amoebae were again counted, the results of which are given in Table II. This table shows the differences in the densities of population still more clearly, for the number of amoebae changes in the alkaline solution only imperceptibly, whereas in the acidic solution it increases considerably. However, it proves that the time of counting the amoebae plays no important rôle in the experiment.

These experiments, as well as the previous ones, show that the appearance of the cysts depends in the first place on the age of the cultures. As the maximal density of population is constant, we can restrict ourselves to the sum totals in our further discussion without considering the relation between amoebae and cysts.

The results gave room for the possibility that by making the nutrient solution alkaline a substance necessary for growth and respiration precipitated out. In order to check this assumption, an

experiment with two parallel series was performed. The nutrient solution was divided into two equal parts. One part was made strongly alkaline, boiled, and filtered, and by adding HCl the desired pH was obtained. NaOH was added to the other strongly acidic part, so that this solution, also, acquired the desired pH. If the substance which precipitated should really be necessary for the growth and respiration of the amoebae, then, by filtering it, distinct differences between the two series should be apparent. However, not only did such differences not appear but, on the contrary, an agreement between the two series, comprising the six pH ranges from 7.6 to 5.5,

TABLE II

THE EFFECT OF H-ION CONCENTRATIONS ON THE DENSITY OF POPULATION IN A TWO-MONTH CULTURE

| | pH | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 8.2 | 7.6 | 7.0 | 6.7 | 6.6 | 6.2 | 6.0 | 5.8 | 5.7 |
| Amoebae......... | 2.1 | 4.8 | 6.7 | 12.2 | 8.6 | 19.0 | 24.9 | 26 9 | 24.3 |
| Cysts............ | 0.5 | 2.7 | 6.2 | 13.8 | 18.6 | 19.8 | 15.5 | 16.5 | 19.6 |
| Total........ | 2.6 | 7.5 | 12.9 | 26.0 | 27.2 | 38.8 | 40.4 | 43.4 | 43.9 |

was observed. This proved that the H-ion concentration alone is the determining factor involved.

The purpose of the third experiment was to determine the limit of the tolerated H-ion concentrations. A nutrient solution of pH 4.8 was tolerated, although the growth-rate of the amoebae slackened perceptibly and the maximum attained was lower than that at pH 6.4. The depth of the zone of growth was apparently the same as that in other acidic cultures. An accurate determination could not be carried out, since the agar particles flaked out to a certain extent in not-inoculated cultures as well. In solutions of higher H-ion concentrations, the amoebae encysted. The attempt to make them grow under anaërobic conditions did not succeed.

## EXPERIMENTS WITH VARIOUS SALT CONCENTRATIONS

A second series of experiments was arranged with nutrient solutions of different salt concentrations. At first the NaCl content was

varied in an otherwise unchanged nutrient solution of pH 6.8. The nutrient solution free of NaCl was boiled, filtered, and divided into two halves. One per cent NaCl was added to one half. The two halves were then mixed in different proportions, so that a series of concentrations of 10, 8, 6, 4, 2, and 0 NaCl per mille resulted. Table III and Figure 2 show the results. They prove conclusively

TABLE III

THE EFFECT OF DIFFERENT NaCl CONCENTRATIONS ON THE DENSITY OF POPULATION OF AMOEBAE

| | NaCl | | | | | |
|---|---|---|---|---|---|---|
| | 1.0 per cent | 0.8 per cent | 0.6 per cent | 0.4 per cent | 0.2 per cent | 0.0 per cent |
| Amoebae | 0.1 | 0.5 | 0.2 | 1.7 | 1.7 | 0.3 |
| Cysts | 1.8 | 1.7 | 1.6 | 12.4 | 15.0 | 14.7 |
| Total | 1.9 | 2.2 | 1.8 | 14.1 | 16.7 | 15.0 |

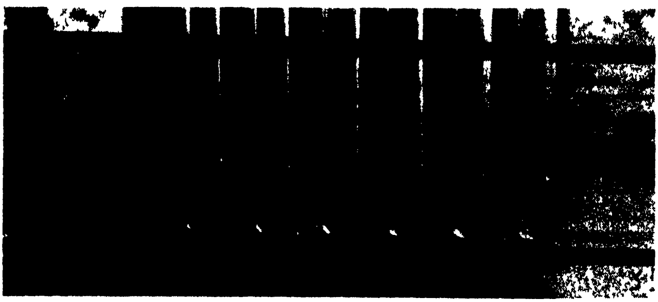

FIG. 2.—The different depths of the zones of growth in various salt concentrations The numbers indicate the NaCl concentrations per mille

that an increase in NaCl concentration has an effect similar to that of an increase in alkalinity, i.e., it causes a dispersed growth, a heightening of the zone of growth, and lowers, at the same time, the maximal density of population.

In other experiments the concentration of all salts was varied in an analogous manner, whereby a tenfold basic solution was the highest concentration used. These experiments yielded similar re-

sults. Finally an experiment was done by replacing the usual solution by artificial sea water of the following composition: 3.0 NaCl, 0.07 KCl, 0.26 $MgSO_4$, 0.50 MgCl, per cent, respectively. One per cent, respectively, peptone and glucose were added to this salt solution. The final solution was diluted with a 1 per cent solution of peptone and dextrose in distilled water; and the following concentrations of NaCl were tried out: 3, 2.4, 1.8, 1.2, 0.6, and 0.3 per cent, respectively, the other salts being diluted in the same proportions. This experiment corroborated the previously obtained results. A rich growth and a distinct formation of a zone of growth were observed only at 0.3 per cent NaCl concentration. The phenomenon is the more curious, as it was shown in a previous paper (Reich, 1933) that *Mayorella palestinensis* showed a rich growth in a nutrient solution containing bacteria, even at 3 per cent NaCl.

### THE EFFECT OF pH AND SALT CONCENTRATION COMBINED

A series of experiments was performed to test the effect of various salt concentrations combined with different H-ion concentrations. Of all these experiments, only one will be reported in detail. The experiment in question dealt with eight nutrient solutions of various NaCl content (3, 2, 1.5, 1.0, 0.8, 0.5, 0.25, 0.0 per cent, respectively), combined with six different H-ion concentrations (pH 7.6, 7.2, 6.6, 6.4, 6.2, 5.8). The results showed that at a NaCl concentration of from 3 to 0.8 per cent the H-ions have no visible effect, and no limited zone of growth appeared in any of these nutrient solutions. It was ascertained, on the other hand, that the NaCl concentration influences the density of population, if only slightly. It amounted at 3 per cent to 0.15, at 2 per cent to 0.3, at 1.5 per cent to 0.87, at 1 per cent to 1.5, and at 0.8 per cent to 1.8 amoebae per 0.1 cu. mm. nutrient solution. With the decrease of the NaCl concentration from 0.5 to 0.0 per cent, the H-ion concentration gained in importance. The latter's effect on the population maximum is clearly seen from Table IV.

These latter concentrations also showed clearly an effect upon the zone of growth. At 0.5 per cent NaCl and pH 7.6, 7.2, and 6.6 a diffuse growth, lacking a pronounced zone of growth, was observed; at pH 6.4 it was observed 5 mm. below the surface; at pH 6.2, 10

mm. below the surface; and at pH 5.8, 15 mm. below the surface. At NaCl concentration of 0.25 per cent, the first indistinct ring formation was observed at pH 7.2. At pH 6.6 a distinct one at a depth of 5 mm.; at pH 6.4, at a depth of 10 mm.; and at pH 6.2 and

TABLE IV

THE EFFECT OF DIFFERENT NaCl CONCENTRATIONS AND DIFFERENT H-ION CONCENTRATIONS COMBINED ON THE DENSITY OF POPULATION OF AMOEBAE

| | pH | | | | | |
|---|---|---|---|---|---|---|
| | 7.6 | 7.2 | 6.6 | 6.4 | 6.2 | 5.8 |
| | 0 5 per cent NaCl | | | | | |
| Amoebae | 1.1 | 1.6 | 1 8 | 4.2 | 2.9 | 13.4 |
| Cysts | 1.1 | 1.4 | 2.4 | 5.2 | 6.1 | 14.5 |
| Total | 2.2 | 3 0 | 4.2 | 9.4 | 9.0 | 27.9 |
| | 0.25 per cent NaCl | | | | | |
| Amoebae | 2.9 | 2.1 | 0.6 | 0.7 | 0.6 | 8.8 |
| Cysts | 1.3 | 3 5 | 4.6 | 9.3 | 12.1 | 38.6 |
| Total | 4.2 | 5.6 | 5.2 | 10.0 | 12.7 | 47.4 |
| | 0.0 per cent NaCl | | | | | |
| Amoebae | 3.5 | 0.3 | 0.3 | 0.4 | 3.8 | 8.8 |
| Cysts | 0.8 | 3.5 | 8.1 | 5.8 | 21.4 | 26.9 |
| Total | 4.3 | 3.8 | 8.4 | 6.2 | 25.2 | 35.7 |

5.8, at a depth of about 15 mm. In the solution free of NaCl, a distinct zone of growth appeared already at pH 7.2, at a depth of 5 mm. In the presence of higher H-ion concentrations, the said zone developed uniformly at a depth of 15 mm.

Summarizing, we can state that in solutions of more than 0.5 per NaCl, the salt concentration has a decided influence upon the

growth, while the H-ion concentration has no effect. At low salt concentrations, however, the effects of both factors sum up.

EXPERIMENTS WITH VARIOUS DEXTROSE CONCENTRATIONS

These experiments proved conclusively that the depth of the ring of growth is not conditioned by the density of population in a purely mechanical way. The different dextrose concentrations attain during the autoclaving of the nutrient solution various degrees of acidity. Therefore the solution was sterilized without dextrose and the desired concentration obtained by adding 0.5 cc. of a correspondingly diluted sterile dextrose solution. Concentrations of 0, 0.5, 1, 2, and 4 per cent, respectively, of dextrose were used. A uniform pH of 5.8 was thus obtained in all solutions. Table V and Figure 3 illustrate the results of these experiments clearly. The density of

TABLE V

THE EFFECT OF DIFFERENT DEXTROSE CONCENTRATIONS ON THE DENSITY OF POPULATION OF AMOEBAE

| | Dextrose | | | | |
|---|---|---|---|---|---|
| | 0.0 per cent | 0.5 per cent | 1.0 per cent | 2.0 per cent | 4.0 per cent |
| Amoebae | 0.2 | 0.1 | 0.3 | 0.0 | 0.0 |
| Cysts | 7.7 | 24.1 | 22.3 | 5.7 | 2.2 |
| Total | 7.9 | 24.2 | 22.6 | 5.7 | 2.2 |

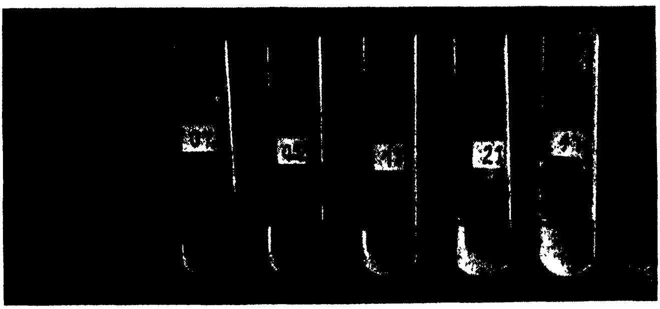

FIG. 3.—The different depths of the zones of growth at various dextrose concentrations.

population remains very low as well at 0 as at 2 per cent dextrose, and reaches its regular density in cultures at 0.5 and 1 per cent dextrose; the zone of growth, on the other hand, is equally deep in the first four concentrations and disappears only at 4 per cent dextrose. Thus, we observe a distinct ring formation at 0 and 2 per cent, despite the low density of population.

## DISCUSSION

When discussing the results, one must naturally make a distinction between the problem of the attained effect on the maximal density of population and that of the effect on the depth of the zone of growth of amoebae. As far as the limits of the maximal population density are concerned, my results confirm the already previously known facts, which were recently found to hold true by Phelps (1935) also for sterile ciliate cultures. What factors determine this limitation is, however, not known yet. It is certainly not a matter of lack of nutritive material. We should rather assume a retarding effect of metabolic products on the division rate of the amoebae, which, under various external conditions, limit the propagation of the amoebae at different densities of population. Further research should aim at clearing up the nature of these division-preventing substances.

It is of interest to notice that some factors, such as NaCl concentration or pH, which affect the sterile cultures to a rather large extent are of only minor importance in cultures containing bacteria. Since the two cultures differ mainly by their manner of nutrition, the assumption that the foregoing factors injure the digestive apparatus of the amoebae, and principally their proteolytic capabilities, appears to be nearest the truth.

Secondly, the experiments show clearly that the amoebae are positive to different oxygen tensions under different external conditions. This makes it difficult to specify the oxygen requirement as a criterion for differentiating between the various species of amoeba. On the basis of the experiments here described, it is impossible to ascertain what factors condition the differences in behavior. The rate at which the once-disturbed stratification assumes its former state shows clearly that the amoebae aggregate actively around the

areas of optimal oxygen tension and that the ring formation consequently does not result from an increase in growth.

Also, the experiments do not ascertain whether the positive reactions to low oxygen concentrations is closely connected with the actual oxygen consumption of the cells and their manner of respiration.


SUMMARY

The effect of changes in the culture medium (pH, salt concentration, and dextrose concentration) upon the maximal density of population and the oxygen tension to which amoebae are positive has been studied. It was shown:

1. The maximal density of population decreases with increasing salt concentration as well as with increasing alkalinity.

2. The maximal density of population is highest at a dextrose concentration of 0.5–1 per cent.

3. In acid nutrient solutions and in those of lower salt concentrations the amoebae are positive to a lower oxygen tension than in alkaline solutions or those of higher salt concentration.

4. The concentration of dextrose from 0 to 2 per cent does not influence the reaction to oxygen tension.


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# Physiological Zoölogy

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# DIFFERENTIAL ACCELERATION IN FROG DEVELOPMENT

(Eight figures)

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ON THE theory of axial gradients, a stimulating agency applied in early development should steepen the gradient and exert a differential acceleration upon apical structures, just as toxic agencies should flatten the gradient and exert a differential inhibition. Of differential inhibition numerous examples are known. For differential acceleration, however, the evidence is less full and mostly applies to invertebrates (for examples of this and of differential inhibition see Child's books [1915, 1924]). The vertebrate cases are confined to the effect of atropine on the fish *Macropodus* (Gowanloch, 1924) and those of weak acids and extremely weak toxic agents (by differential acclimatization) on frogs, reported by Bellamy (1919, 1922).

Gowanloch's work has unfortunately never been published *in extenso* and cannot be evaluated. Bellamy found definite acceleration of rate of development by the application to early stages of very weak concentrations of various normally toxic substances. He especially notes n/500 NaOH, 8 days from early gastrula; m/100,000 KCN, 4 days from fertilization; n/5,000 and n/20,000 HCl for 9 days from early cleavage. This absolute acceleration was accompanied by the formation of relatively large heads (differential acceleration in Child's sense).

Professor Child, in a letter, suggested the use of pilocarpine. This had been found by Matthews (1902) to accelerate development in sea urchins. Sollman (1904), working with sea-urchin and starfish eggs, confirmed this accelerating effect for low concentrations of pilocarpine, though high concentrations retard. Atropine in low concentrations has no effect; in higher concentrations it has a marked retarding effect. Pilocarpine further was found by Hinrichs (1929) and Hinrichs and Gunther (1929) to have a slight accelerating action on cleavage in *Arbacia* and a marked differential stimulative action on apical regions, whereas atropine was highly inhibitory and caffeine gave negligible results. Unfortunately, Hinrichs' paper is only a note, and gives no detailed protocols or figures. Earlier, Hinrichs (1924) had studied the effect of caffeine on *Planaria*, with especial regard to oxygen consumption, and found in general that low concentrations caused a marked increase, followed later by a decrease, in metabolism. Hinrichs' results with pilocarpine and caffeine, though scrappy, are suggestive.

In view of the great advances of recent years in the analysis of vertebrate development, we decided to repeat some of these experiments on frog's eggs (*Rana temporaria*). The present paper, though largely in the nature of a preliminary communication, records some definite results, notably with pilocarpine and HCl.

## METHODS

After artificial fertilization, the frog's eggs were placed, either immediately or sometimes in later embryonic stages, for a certain period (mostly 1–2 days) in various solutions of caffeine, atropine, pilocarpine, ethyl alcohol, potassium cyanide, lithium chloride, and hydrochloric acid, respectively. At certain stages of larval life (mostly 7–10 days and 5–6 weeks) samples of tadpoles were taken from the various solutions and preserved in Bouin's alcoholic fluid. These samples were drawn later in ventral and in lateral view on millimeter paper with the aid of a projection apparatus (constructed by Mr. D. A. Kempson), which gave a magnification of exactly 12×. From the sketches the proportions of the larvae were calculated and tabulated in order to compare the effect of the various solutions upon the size and proportions of the larvae. It should be mentioned that the

data obtained from the sketches were not recalculated into millimeters; thus the units given in the tables and text are 1/12 millimeter each. Five measurements were taken from each sketch, as follows (see also Fig. 1): (1) maximal head breadth, as seen in ventral view; (2) maximal head length, determined arbitrarily as the distance from the extreme anterior end to the most posterior level of anterior gill insertion, as seen in ventral view; (3) maximal trunk breadth, as seen in ventral view; (4) trunk length, as measured from the anterior end of the head to the posterior level of the orifice of the anal tubule, as seen in lateral view; (5) total length, as measured in lateral view from the anterior end of the head to the posterior end of the tail, along the main axis of the body (*not* the shortest distance in cases where the body is bent).

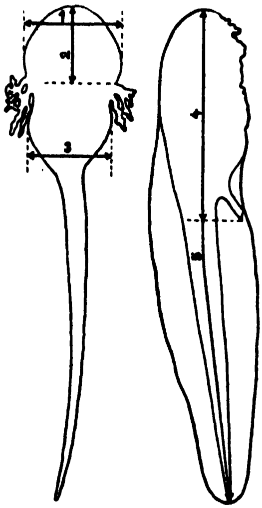



FIG. 1.—Outline sketch of 8-day frog larva in ventral (*a*) and in lateral (*b*) views, showing measurements made (indicated by double arrows): 1, head breadth; 2, head length; 3, trunk breadth; 4, trunk length; 5, total length.

A preliminary analysis of the data so obtained showed that two main ratios can be calculated from them which seem to be characteristic for the larvae. The one is the head-breadth/head-length ratio; the other the ratio of either total length to head length, or, what is essentially the same but gives more marked results, "body length" (as obtained by subtracting head length from total length) to head length. A third ratio which we first thought to be of importance, i.e., trunk length to head length, turned out to be of little value, because the trunk length shows a great fluctuating variation, which seems to bear no relation with other proportions. A few individual measurements are given in Table I.

It soon turned out, however, that the differences between the samples treated with various solutions are too slight to override individual variability. Thus, only mass methods were applicable, and the results here reported are based on averages of a number of measurements. Owing to this circumstance, not all of our sample stocks were

available for analysis, but only those which contained fairly high numbers of individuals. From the three series of our experiments, the third series (Exp. 13/4/33), which was in many respects the most elaborate, has been included *in extenso;* and practically all individuals from it were drawn, except those which were obviously abnormal (e.g., larvae with tumorous outgrowths, etc.) or were damaged during preservation. In this series the effects of pilocarpine and of hydrochloric acid were studied. In addition some stocks of the first series of experiments (Exp. 3/4/33) were studied in detail, i.e., those treated with caffeine, atropine, and potassium cyanide; but the numbers preserved were unfortunately too small to allow of definite conclusions being safely drawn. The second series of experiments (Exp. 10/3/33) has been used for auxiliary purposes only.

TABLE I

| Head Breadth | Head Length | Trunk Breadth | Trunk Length | Total Length |
|---|---|---|---|---|
| KCN, m/100,000, 9-Day Larvae | | | | |
| 23.5 | 19.0 | 21.0 | 57.5 | 123 |
| 26.0 | 22.5 | 22.5 | 57.5 | 128 |
| 27.0 | 22.5 | 24.5 | 56.5 | 141 |
| 28.5 | 25.5 | 26.0 | 61.0 | 145 |
| 29.5 | 25.5 | 26.5 | 59.5 | 146 |
| 32.0 | 27.0 | 29.0 | 58.5 | 151 |
| HCl, m/5,000, 8-Day Larvae | | | | |
| 20.0 | 14.0 | 17.5 | 56.0 | 110 |
| 21.5 | 15.0 | 20.5 | 61.0 | 115 |
| 22.0 | 15.5 | 21.0 | 58.5 | 124 |
| 23.5 | 15.5 | 21.5 | 57.5 | 127 |
| 23.5 | 15.5 | 21.0 | 55.5 | 128 |
| 26.0 | 18.5 | 24.5 | 55.5 | 138 |
| 26.5 | 18.5 | 25.0 | 60.5 | 139 |

## RESULTS

*a*) *Effects on rate of development.*—Certain agents definitely accelerated the rate of development above that of the controls, while others delayed it. Delay was noted with LiCl and alcohol and the

stronger concentrations of KCN. Very weak KCN sometimes caused slight acceleration. Certain concentrations of HCl and of pilocarpine caused definite acceleration. This was most pronounced with m/10,000 HCl (24 hours' exposure) and 1/4,000 pilocarpine (24 hours' exposure) (both from first cleavage); m/5,000 HCl (24 hours) and m/10,000 HCl (48 hours) were also accelerated, but to a lesser degree. This was also true for pilocarpine 1/4,000 (48 hours) and 1/2,000 and 1/8,000 (24 hours).

For some reason the acceleration caused by HCl in Experiment 10/4/33, though distinct, was only slight, and not nearly so marked as in the other two experiments. The acceleration caused by HCl and pilocarpine was visible after 2 days; but after 7 days and over it was more obvious in the HCl series, while the pilocarpine larvae became characterized by relatively larger heads (see below).

Thus a distinct absolute acceleration of development was caused by the same two agents which, as will be seen later, caused differential acceleration in the sense of a relative increase in the size of certain anterior measurements. This confirms Bellamy's work. However, as will be seen, the type of differential acceleration was not the same in HCl and atropine, so that we cannot put down the changes of proportions to a purely non-specific quantitative factor expressing itself also in acceleration of development.

*b*) *Effects on proportion of parts.*—Beginning with the most numerous data, i.e., those concerning the 8-day larvae of Experiment 13/4/33, one finds the following regularities (see Table II):

1. The normal head-breadth/head-length ratio is between 1.27 and 1.36, and the normal body-length/head-length ratio is between 6.6 and 6.7. Both the stocks kept in the weakest HCl and pilocarpine solutions give these ratios. Unfortunately, owing to inadequate numbers of the controls, no controls were preserved at 8 days: controls were, however, preserved at 7 days (see below). However, the fact that the weakest HCl and pilocarpine 7-day stocks give closely similar results, which then diverge in various particulars in higher concentrations, is evidence that these weak solutions had only slight effects.

2. The stronger HCl stocks seem to keep the normal body-length/head-length ratio (i.e., the relative head length does not vary with

TABLE II

SUMMARY OF EXPERIMENT 13/4/33, 8-DAY LARVAE

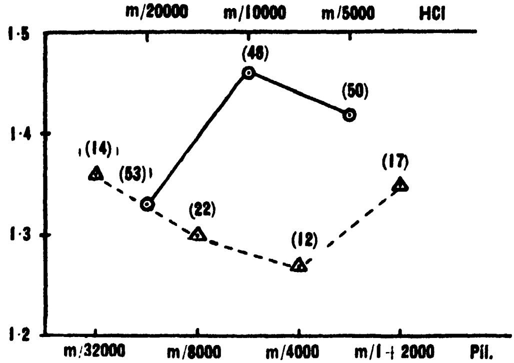
| | No. of Specimens | Head Breadth | Head Length | $\sqrt{\text{Head Breadth} \times \text{Head Length}}$ | Body Length | Total Length | $\frac{\text{Head Breadth}}{\text{Head Length}}$ | $\frac{\text{Body Length}}{\text{Head Length}}$ | $\frac{\text{Body Length}}{\sqrt{\text{Head Breadth} \times \text{Head Length}}}$ |
|---|---|---|---|---|---|---|---|---|---|
| HCl: | | | | | | | | | |
| m/5,000 | 50 | 22.8 | 16.1 | 19.15 | 107.9 | 124 | 1.42 | 6.70 | 5.63 |
| m/10,000 | 46 | 23.1 | 15.8 | 19.10 | 105.2 | 121 | 1.46 | 6.66 | 5.51 |
| m/20,000 | 53 | 22.8 | 17.2 | 19.80 | 113.8 | 131 | 1.33 | 6.60 | 5.75 |
| Pilocarpine: | | | | | | | | | |
| m/1,000–2,000 | 17 | 22.2 | 16.5 | 19.14 | 101.5 | 118 | 1.35 | 6.12 | 5.30 |
| m/4,000 | 12 | 23.5 | 18.6 | 20.90 | 107.4 | 126 | 1.27 | 5.84 | 5.14 |
| m/8,000 | 22 | 23.6 | 18.2 | 20.72 | 111.8 | 130 | 1.30 | 6.14 | 5.39 |
| m/32,000 | 14 | 24.2 | 17.7 | 20.67 | 118.3 | 136 | 1.36 | 6.68 | 5.72 |

HCl treatment), whereas the head-breadth/head-length ratio markedly increases in more concentrated solutions (1.42–1.46). In other words, the head shape tends to become broader under the influence of hydrochloric acid.

3. On the contrary, in the pilocarpine stocks the normal head-breadth/head-length ratio is approximately constant in the various concentrations, the fluctuations being within the limits of probable error, i.e., the shape of the head does not vary with pilocarpine treatment. On the other hand, the body-length/head-length ratio definitely decreases in the stronger solutions (from 6.7–6.8 to 5.8–6.1), which means that the relative length of the head increases with pilocarpine treatment (see also Figs. 2–7).

A measure of the size of the head as a whole may be arrived at by taking the square root of the product of head breadth times head length. The ratio of body length to this then gives a measure of relative head size. For this, the lowest concentrations of both HCl and pilocarpine again give approximately equal values: relative head size then increases slightly with greater concentration of HCl but markedly with greater concentration of pilocarpine.

Table II and the graphs (Figs. 2–7) bring out the following further points. Increasing concentrations of pilocarpine cause: (1) a progressive decrease in absolute total length (Fig. 7); (2) a progressive decrease in absolute body length (Fig. 5); (3) an increase (followed by a slight decrease) in absolute head length (Figs. 5, 6); (4) a slight progressive decrease in absolute head breadth (Fig. 5); (5) a progressive increase (followed by a marked decrease) in absolute head size; (6) a marked increase (followed by a final decrease) in relative head length (Fig. 3); (7) a definite decrease (followed by an increase) in relative head breadth (Fig. 2); (8) a marked increase (followed by a decrease) in relative head size (Fig. 4). Increasing concentrations of HCl, on the other hand, cause an irregular decrease in (9) total length (Fig. 7), (10) in absolute body length (Fig. 5), (11) in absolute head length (Figs. 5, 6), and (12) in absolute head size; and (13) no decrease or a slight increase in absolute head breadth (Fig. 5); they also cause (14) a negligible progressive decrease in relative head length (Fig. 3), (15) a definite but irregular increase in




FIGS. 2–7.—Effects of HCl (continuous line) and pilocarpine (dotted line) on 8-day larvae, experiment 13/4/33. In all figures, the groups treated with m/2,000 and m/1,000 pilocarpine have been combined, and the abscissae represent concentrations.

FIG. 2.—Effect on relative head breadth. Ordinates, head-breadth/head-length ratio.

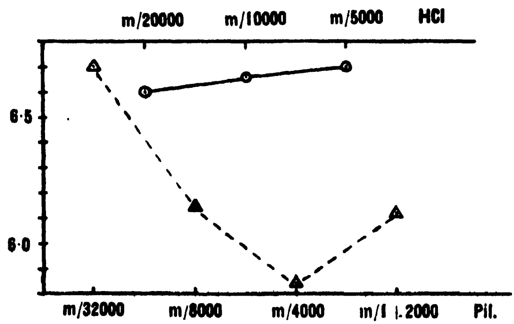



FIG. 3.—Effect on relative head length. Ordinate, body-length/head-length ratio

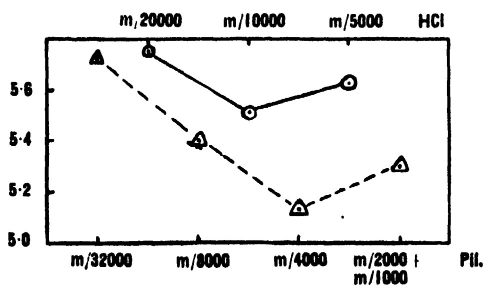



FIG. 4.—Effect on relative head size. Ordinate, body-length/head-breadth × head-length ratio.

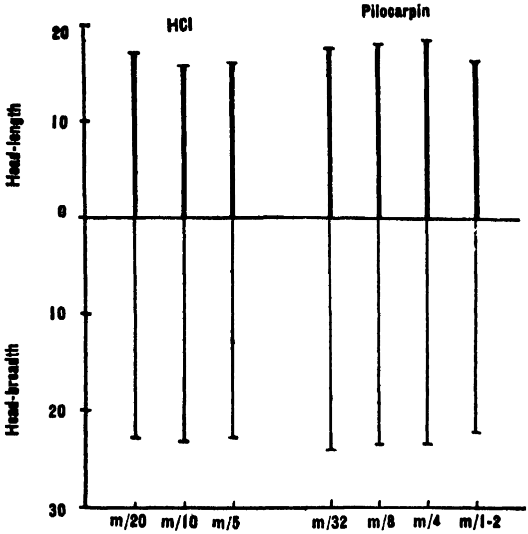



FIG. 5.—Absolute measurements of head length and head breadth. In this and Figures 6–7 the units on the ordinate each represent 1/12 mm.; and all concentrations are to be multiplied by $10^{-3}$.

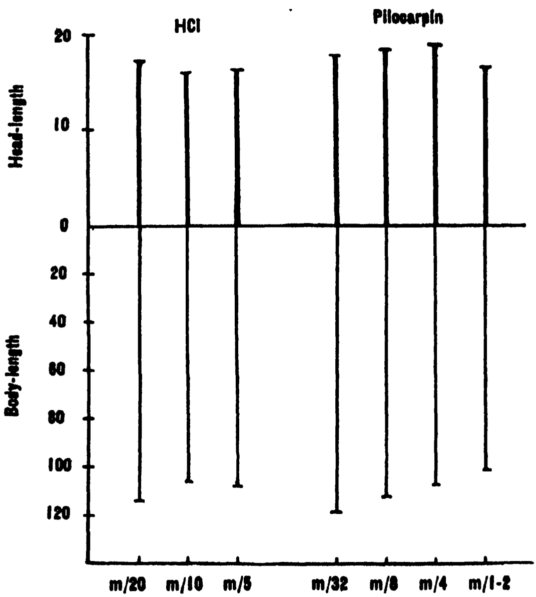



FIG. 6

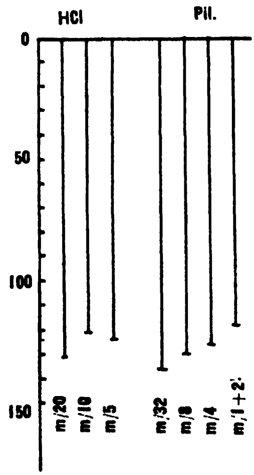



FIG. 7

FIG. 6.—Absolute measurements of head length and body length (= total length − head length).

FIG. 7.—Absolute measurements of total length

relative head breadth (Fig. 2), and (16) a similar but less marked change in relative head size (Fig. 4).

The differential acceleration exerted by pilocarpine on the head is well shown by the progressive increase of head length and negligible decrease of head breadth, the two combined giving progressive increase of absolute head size, accompanying definite decrease of body length, in the first three concentrations. The highest concentrations (m/2,000+m/1,000) appear to have been overstrong and to have begun to exert a general inhibitory effect.

Similarly, the increased head breadth in m/10 HCl accompanying decreased head length and decreased body length well shows the effect of this agent upon head growth in breadth.

Besides these 8-day larvae, small stocks (7–9 specimens each) of 7-day larvae were also preserved in this experiment. These stocks contain the most advanced larvae of various concentrations, and thus cannot be directly compared with the much more numerous and random samples of 8-day larvae. One point, however, should be noted, which is manifested by these larvae, as well as by others to which we shall refer later; this is, that in general the shape of the head alters with age. In older larvae the relative length of the head definitely increases, and thus both the body-length/head-length and the head-breadth/head-length ratios gradually decrease. This is apparently due to the formation of the internal gill system during larval life. As a result of this, we find in the 7-day larvae somewhat greater values for both the body-length/head-length and head-breadth/head-length ratios than in the 8-day larvae (6.9–7.4 as against 6.6–6.7, and 1.37–1.49 as against 1.33–1.36).

As regards the effects of chemical agencies, the results from these 7-day larvae, so far as they go, confirm those found in the 8-day samples.

Thus, the average head-breadth/head-length ratio is markedly increased in comparison with the control stock (1.49 for HCl, 1.45 for pilocarpine, 1.37 for controls). The body-length/head-length ratio is, however, inconclusive, as it is smaller in the pilocarpine but bigger in the HCl stock than in the controls (6.85 for pilocarpine, 7.03 for controls, 7.42 for HCl). It must be repeated that the numbers are too small to be conclusive. The same applies to the 5-week

larvae, which were also preserved in very small numbers (4–8 in each sample) on the erroneous supposition that the differences would be clear-cut enough to be demonstrable on any treated specimen. However, it is noteworthy that in these larvae the body-length/head-length ratio is as low as 4.7–4.9, in spite of the increase of total length to 180–95 units; and the head-breadth/head-length ratio is only 1.25–1.33, which thus confirms our statement as to the change of shape of the head with age.

FIG. 8.—Ventral view of larvae of comparable development, showing the effect of various treatments. From left to right: 1, control; 2, treated with HCl—broad head; 3, treated with pilocarpine—large head; 4, treated with atropine—long head.

The caffeine, atropine, and KCN stocks of Experiment 3/4/33[1] were, as noted, statistically insufficient. Further, owing to higher temperatures, the 8-day larvae of this experiment were further advanced in development than those of the same temporal age in Experiment 10/4/33, making direct comparison impossible. In general, however, it may be stated that all these treatments, notably those with the weakest solutions of KCN, increased relative head length.

[1] Caffeine m/2,500; atropine m/10,000 and m/50,000; 24 hours from dorsal lip; KCN m/10,000 for 2 days and m/100,000 for 4 days (renewed daily) from 1st cleavage.

This is evident when samples of about the same age (as judged by the gills) are preserved and compared (see Fig. 8). Further, when the treated 8-day larvae of this experiment are compared with the 5-week larvae of other experiments, it is found that their head-breadth/head-length ratio is lower instead of higher, thus forming an apparent exception to the rule that older animals have relatively longer heads, which can only be due to these larvae having unusually narrow, long heads for their age. In the 10/4/33 series, the weaker KCN (m/50,000 and m/10,000, 24 hours from first cleavage) were also markedly long-headed, while stronger solutions (m/4,000 and m/8,000) were definitely, if slightly, inhibited in all dimensions.

## CONCLUSIONS

Apart from stating that distinct evidence of some differential action on head form is apparent, together with evidence that in some cases absolute acceleration of development occurs, no profitable discussion of the results with atropine, caffeine, and KCN can at this stage be attempted. The pilocarpine results, on the other hand, appear to be conclusive. Pilocarpine in concentrations of m/8,000 and m/4,000 exerts a differential stimulation on the head region, causing this to enlarge both absolutely and relatively, even though it produces a decrease in absolute body length. At concentrations above m/4,000 the stimulating effect gives place to inhibition, confirming Hinrichs and other workers.

The results with HCl are not so marked as those recorded by Bellamy. There is, however, a distinct change in the shape of the head in the direction of increased breadth, and a slight but definite increase in relative (but not absolute) head size. In both cases this effect is greatest at m/10,000. It should also be noted that our treatment lasted only 1 or 2 days, while Bellamy, using similar concentrations, obtained his best results by leaving the eggs and embryos in the solutions for 9 days.

It is important to recall that the treatment was applied in early stages (usually from first cleavage, sometimes from early gastrula) and that the effects only became visible much later. Either the gradient system was altered (in the egg as a whole or in the indi-

viduation field of the organism) or the activity of the organizer was modified in some other, as yet unascertained, manner.

It is proposed, now that we have gained experience as to suitable substances, concentrations, times of treatment, etc., to repeat the work on a larger scale on fish material.

SUMMARY

1. Pilocarpine, HCl, and apparently also atropine, caffeine, and KCN, administered in weak solutions to developing frog's eggs for 18–48 hours in early stages (usually from first cleavage, sometimes from early gastrula), cause various differential accelerations in development.

2. Pilocarpine accelerates the development of the head as a whole; HCl accelerates the development of head breadth; whereas KCN, atropine, and perhaps also caffeine, seem to accelerate the development of head length.

3. The effects are clearly shown in 8-day larvae but possibly persist in later stages also.

4. In general the relative length of the head increases during larval development, owing to the formation of the internal gill system.

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# EXPERIMENTAL ASYMMETRIES OF THE HEAD OF EUPLANARIA DOROTOCEPHALA[1]

(Twelve figures)

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THE remarkable capacity of pieces of planarians in reconstituting whole animals long ago aroused the curiosity and interest of various investigators. The result has been that many reports dealing with the regulation of pieces of different sizes and shapes from various species are to be found in the literature. In general it has been found that pieces of planarians above a certain minimum size and regardless of shape reconstitute, after a time, into perfectly symmetrical individuals, providing the species possesses the capacity for reconstitutional development.

It is true that short transverse pieces, particularly from the more posterior regions of the anterior zooid, in *Euplanaria* commonly give rise to headless individuals or individuals possessing various types of inhibited heads (Child, 1911); but this phenomenon has been shown to be the result of an inhibitory factor arising from posterior section (Child and Watanabe, 1935). In other words, all levels of *Euplanaria* will develop normal heads if the head-forming cells of the anterior cut surface are not exposed to influences arising from posterior section or other inhibiting conditions. Certain inhibiting agents have been used to block the activity of the posterior cut surface in short transverse pieces of *E. dorotocephala*, thereby inducing an increase in the head frequency at levels in which the head frequency would normally be very low (Child, 1916; Buchanan, 1922; Hinrichs, 1924).

In the regulation of pieces toward a normal bilateral symmetry it has been found that the polar relationships within a piece may be altered during the reconstitutional process. One of the best examples of this is given in the work of Beyer and Child (1930), in which it was

[1] This investigation was supported by funds from a grant by the Rockefeller Foundation in aid of research in the biological sciences at the University of Chicago.

shown that short transverse pieces of *Euplanaria*, half the width of the body or less, may develop heads on the anteromedial angle or the medial cut surface of the piece instead of on the anterior cut surface. In spite of the inhibition of heads and the alteration of polarity, the animals which reconstitute generally attain a bilateral symmetry; and it is with symmetry in reconstitution that this paper deals.

## THEORETICAL CONSIDERATIONS

Morgan (1901) was much interested in the fact that the reconstituting head in *Planaria lugubris* (according to Hyman, 1931, this species is probably *Curtisia foremanii*) did not arise in the middle of an oblique-anterior cut surface. Instead it appeared, in its early development, to be restricted more or less to the more anterior region of the cut surface. The new head was usually asymmetrical in that the side which developed nearest the longer margin of the body gave rise to an eyespot before the more posterior side. There seemed to be even more asymmetry in the reconstitution of an animal from an oblique strip (made by two parallel oblique cuts) than in the reconstitution from a piece with only an oblique-anterior cut surface.

Rand and Boyden (1913), in working on the inequality of the two eyes in heads arising from an oblique-anterior cut surface in *Planaria maculata*,[2] noted a tendency of the inner eye to be smaller than the outer eye. It was found in this work that the animal curved toward the shorter side after the anterior end was removed obliquely. The eye which appeared on the side of the head arising on the shorter side of the animal was referred to as the "inner" eye, the "outer" eye being, of course, the eye which appeared on the side of the head arising on the longer side of the animal. They also found in a few cases that there was a tendency for the size of the eyes of the two sides to be reversed if a longitudinal strip was removed from the outer side of the reconstituting animal. The removal of this strip resulted in a reversal of body curvature, particularly in the more posterior regions of the piece. Rand and Boyden do not attempt to give a definite explanation for this temporary inequality of the two eyes, but they

[2] Hyman (1931) would probably designate this species as *Euplanaria maculata* or *E. novangliae.*

do say that the positional relations of the larger and smaller eye are closely correlated with the form of the regenerating piece.

Watanabe (1935*a*) has shown that different levels in *Euplanaria dorotocephala* develop heads at different rates. In the anterior zooid the more anterior levels develop eyespots before the more posterior levels of the same zooid. (These experiments were conducted with pieces long enough to give normal heads.) In experiments such as those described by Morgan (1901) and by Rand and Boyden (1913) with an oblique-anterior cut surface alone, it is readily seen on the basis of Watanabe's findings why the more anterior (outer) side of a head developing from an oblique-anterior cut surface should develop an eyespot before the more posterior (inner) side.

Child and Watanabe (1935), in attempting to determine the relationships between anterior and posterior cut surfaces of short transverse pieces of *Euplanaria dorotocephala*, gave considerable attention to a particular series of experiments which throws much light on temporary asymmetry in the reconstitution of a new head. They found that in the posterior half of the animal asymmetry in the head developing from the cut surface could be induced by imposing a posterior half-section a short distance behind the anterior whole section. The asymmetry of the head appeared as a temporary inhibition in size of eyespot and auricle as well as in amount of new tissue on the side of the head affected by the posterior half-section. When a single posterior operation was made, only a small number developed asymmetrical heads (19 per cent); but when the wound was reopened shortly after healing, 43.5 per cent of the animals developed asymmetrical heads. If a block of tissue was removed at the site of the posterior half-section, 81 per cent of the heads were asymmetrical. The data presented by these authors and by Watanabe (1935*b*) on *E. maculata* suggest that the posterior cut surface acts in preventing the dedifferentiation and activation of the head-forming cells at the anterior cut surface by nervous transmission of the inhibiting factor. In the making of posterior half-sections, as in the experiments above, only one of the ventral nerve cords was affected, with a resulting inhibition of only one side of the reconstituting head.

It is clear from the foregoing investigations that two factors of an entirely different nature may act in producing asymmetry in the

reconstituting head of *Euplanaria*. The first deals with the fact that in the anterior zooid there is an anteroposterior gradient in rate of eyespot formation which clearly indicates why an oblique-anterior cut surface with different head-forming rates along its expanse should give rise to an asymmetrical head. The second deals with the inhibition of anterior growth by certain factors brought into play through the invoking of posterior section. With these two separate factors operating on the symmetry of the head, the problem arises as to whether or not the asymmetry of the head developing from an oblique-anterior cut surface could be antagonized or reversed by imposing a posterior cut surface behind its anterior, more active region.

## MATERIAL AND METHODS

In these studies 14–16-mm. *Euplanaria dorotocephala* were used exclusively. They were collected in fresh-water springs near Chicago several weeks before being used in the experiments. They were not fed for at least 1 week before the experiments were begun. In the laboratory the water on the worms was changed on alternate days.

The test and control lots were always selected from the same stock and cared for identically. After section the animals were kept in finger bowls (25–30 per bowl) in well water until the proper stage for observation was reached. Observations were usually made with a binocular microscope but were often checked, particularly in the early stages, by means of a compound microscope.

The worms were sectioned with a very sharp straight-edged scalpel in shallow water on a glass surface. All cuts were made with a single movement of the knife to avoid jagged edges to the wound. An attempt was always made to make the cuts while the animal was fully extended.

## EXPERIMENTAL DATA

1. *The effects of oblique-posterior half-section on head formation at an oblique-anterior cut surface.*—Three experiments, involving 105 animals, were performed in which an oblique-anterior section was made at an angle of 60° from the transverse axis of the body—the left side being always the more anterior (Fig. 1).[3] It was found that

[3] Oblique sections made at an angle of 45° from the transverse axis failed to give definite results as regards asymmetry.

section could be made with greater accuracy if the head was first removed by a transverse cut directly behind the auricles. These cases of single oblique section are called "controls." Equal numbers

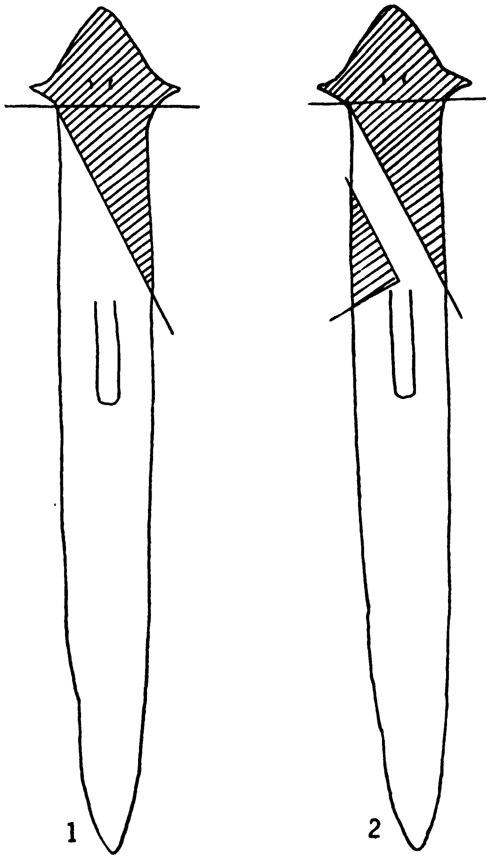



Fig. 1.—Control animals with an anterior-oblique section made at an angle of approximately 60° from the transverse axis of the body.

Fig. 2.—Test animals with a block of tissue removed posterior to the anterior region of the anterior-oblique cut surface.

of animals of the same size and from the same stock were sectioned obliqely in a manner identical with that of the controls. In addition a second oblique section was made at a distance of approximately 1 mm. posterior to the first and running parallel to the more anterior

region to the mid-line of the body. A third section was made at right angles to the second in such a way that a triangular block of tissue was removed from the left side of the animal and a posterior cut surface was created behind the anterior half of the oblique-anterior cut surface (Fig. 2). These animals are referred to as "tests."

The controls and tests were examined 5–6 days after the operations. The symmetry or asymmetry of the head, as well as any other developmental irregularities, was recorded. The animals were then returned to the finger bowls and observed at a later period (12–13 days after section). In Table I are recorded the data for the tests and controls at the different times of observation. As the eyes have been selected as an index to symmetry, the data are presented in comparative size of eyespots.

TABLE I

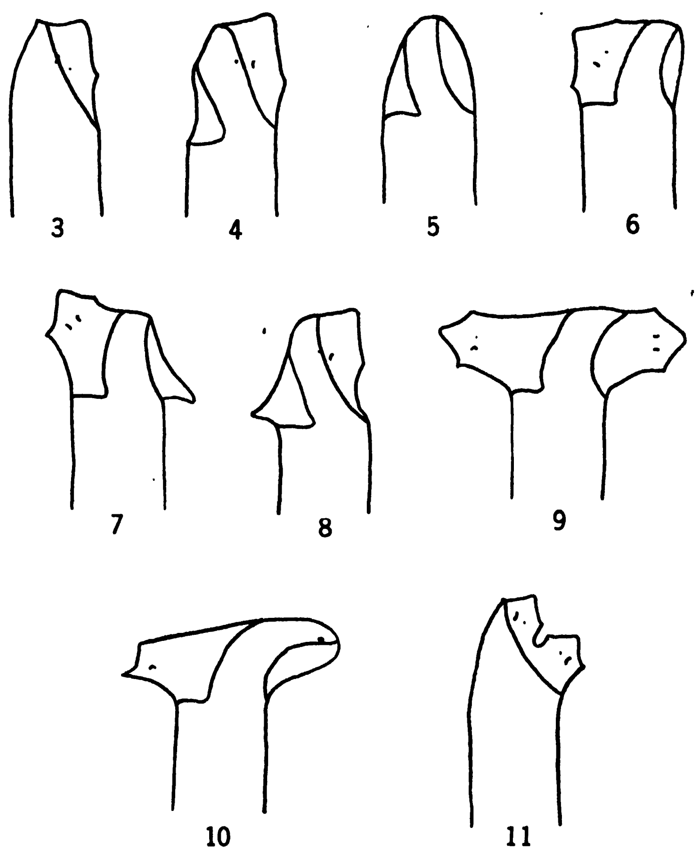
THE EFFECT OF OBLIQUE-POSTERIOR HALF-SECTION ON THE SYMMETRY OF THE HEAD APPEARING ON AN OBLIQUE-ANTERIOR CUT SURFACE

| | 5–6 Days after Section | | 12–13 Days after Section | |
|---|---|---|---|---|
| | Control | Test | Control | Test |
| Small eye, left | 8 | 21 | 21 | 24 |
| Eyes equal | 24 | 17 | 69 | 52 |
| Small eye, right | 70 | 24 | 6 | 13 |
| One eye | 0 | 9 | 0 | 3 |
| Three eyes | 0 | 0 | 8 | 0 |
| No eyes | 2 | 34 | 0 | 12 |

It will be noted in Table I that 5–6 days after section the controls have reconstituted 78 asymmetrical heads, and that in 70 the larger eye is on the left (more anterior) region of the head (Fig. 3). The test worms present quite a different picture, as out of 45 cases of unequal eyes 21 appeared with the larger eye on the right (Fig. 4).[4] Equally as important as the reversal in size of eyespots is the fact that 9 of the test worms show a single eye while in the controls none

[4] The eyes of the different animals shown in the figures are not drawn exactly to scale. When speaking of a reversal in eyespot size in the early observations, it is not meant to imply that the inner eye grows faster but that the outer eye is retarded.

are in this class. Moreover, 34 of the tests at this period in reconstitution show no eyes (Fig. 5) while all but 2 of the controls have developed definite eyespots. It seems quite clear from these data that the oblique-posterior half-section is effective not only in revers-




FIGS. 3–11.—Different forms arising in response to anterior-oblique section (with and without posterior half-section).

ing the difference in size of the eyes but in retarding the development of the head at the anterior cut surface.

After the pieces have reconstituted 12–13 days (Table I) the controls have regulated considerably toward the bilateral symmetry of the species. Sixty-nine out of 104 have eyespots which are, as far as

could be detected, equal. Only 6 of the 70 animals which began with the larger eye at the left have remained in this condition. Instead, of 8 individuals showing the larger eye on the right, as was the case at 5–6 days after section, 21 animals now show the larger eye on the right side. At this time 8 animals show 3 eyes, which suggests that a new eye has arisen on the right in addition to the smaller slightly misplaced eye which first appeared there.

Of the test animals after 12–13 days, 52 show eyespots approximately equal in size, compared to 17 recorded at the earlier stage of observation. Three still show a single eye, and 12 show no eyespots in the small amount of tissue at the oblique-anterior cut surface. Of these 12 with extremely inhibited anterior reconstitution, 8 closely resemble Figure 5, in which no head develops at any region. In 2 a secondary head has developed in the region where the posterior block of tissue was removed with complete inhibition of the oblique-anterior surface (Fig. 6); and in 2 a secondary head has also developed but a tail has been induced on the anterior cut surface, where a head would otherwise appear (Fig. 7).

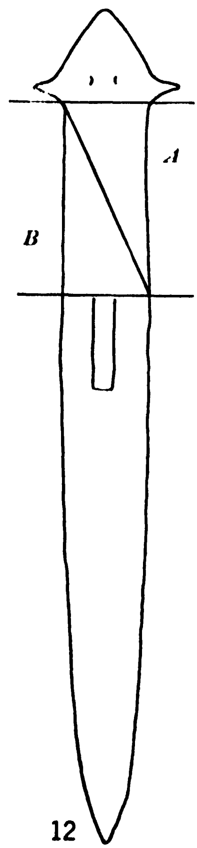



FIG. 12.—*A*, pieces with anterior-transverse and posterior-oblique cut surfaces. *B*, pieces with anterior-oblique and posterior-transverse cut surfaces.

In any work dealing with the sectioning of *Euplanaria*, forms appear which are of interest to the student of reconstitutional development. In fully one-half the test worms a tail appeared at the region where the posterior block of tissue was removed (Fig. 8). In one case (classed as "eyes equal") a head appeared on both the oblique-anterior cut surface and in the region where the block of tissue was removed (Fig. 9). It is interesting to note that in this case the inhibited eye is on the side of the secondary head which springs

from the oblique-posterior cut surface. One form appeared (classed under the heading of "one eye") in which the head arising from the oblique-anterior cut surface is strongly inhibited while the secondary head is very asymmetrical with almost complete inhibition of its "anterior" side (Fig. 10). One atypical form appeared in the controls (Fig. 11) which demonstrated a mutual inhibition of the "inner" eyes when two heads arise on the same cut surface.

2. *The effect of oblique-posterior with transverse-anterior cut surfaces and oblique-anterior with transverse-posterior cut surfaces on the symmetry of the reconstituting head.*—An experiment was performed involving two lots of 25 worms each to demonstrate further the effect

TABLE II

THE EFFECT OF OBLIQUE-POSTERIOR AND TRANSVERSE-ANTERIOR CUT SURFACES AND THEIR RECIPROCALS ON THE SYMMETRY OF THE NEW HEAD

| | *A* | *B* |
|---|---|---|
| Small eye, left | 58 | 2 |
| Eyes equal | 25 | 14 |
| Small eye, right | 0 | 64 |
| Teratophthalmic | 2 | 2 |
| Anophthalmic | 0 | 2 |
| Deaths | 12 | 16 |

of the posterior cut surface in controlling the symmetry of the head. The worms were sectioned in the manner shown in Figure 12. The head and region posterior to *B* were not used in these experiments. Pieces *A* and *B*, together, make up approximately one-fourth the length of the animal. Since the results obtained from the two lots were nearly identical, they are grouped together in Table II in percentages. Shortly after section the cut ends of the pieces contract, giving the piece an asymmetrical appearance. The pieces were examined and classified on the basis of eyespot size approximately 2 weeks after section. Because of the greater area of injury there was a greater number of deaths than is usually found in reconstituting pieces of *Euplanaria*.

The results are unmistakable. Not a single time does the smaller (inhibited) eye appear on the right in *A* pieces. As *A* pieces possess

a transverse-anterior cut surface, it seems evident that the obliquity of the posterior cut surface is alone responsible for the asymmetry of the head developing in this case. In *B* pieces the posterior influence and the body-level (due to oblique-anterior section) unite in producing asymmetry of the head. It will be noted that only 14 per cent developed with equal eyes and that only 2 per cent showed reversal. Comparison of these data with an oblique-anterior cut surface alone (Table I) show the importance of the posterior factor. With an oblique-anterior cut surface alone, a regulation takes place early, so that within 2 weeks after section the asymmetry has disappeared or been reversed.

## DISCUSSION

The gradient hypothesis seems at present to provide the most satisfactory basis for the interpretation of these experiments on asymmetry of the reconstituting head in *Euplanaria*. In the first place, heads developing from an oblique-anterior cut surface show asymmetry in that the more anterior region of the cut surface develops an eyespot first. This fact points to an anteroposterior differential in activity within the anterior zooid. Watanabe (1935*a*) has shown quite clearly that there actually is a gradient in rate of eyespot formation in heads developing from transverse cuts at different levels along the main axis of the animal. Oblique-anterior cuts of the order used in the experiments give an anterior cut surface which is graded in eye-forming rate throughout its expanse. It is therefore to be expected that the most anterior part of an oblique-anterior cut surface should give rise to its portion of a head before the less active more posterior part.

From the onset of reconstitution of the head from an oblique-anterior cut surface there is, however, a gradual regulation toward the bilaterality of the species. This is shown rather diagrammatically in Figure 3. The part of the head arising from the most anterior region of the anterior cut surface is, in reality, made over from old tissue. The auricle and eyespot usually appear in the old tissue. In these experiments the right (more posterior) region of the head is made up entirely of new unpigmented tissue. The later growth of

this new tissue is more rapid on the right than on the left sides of the head, and symmetry usually results.

With the removal of a block of tissue posterior to the anterior half of the oblique cut surface a new set of factors is imposed upon the reconstituting head. These factors tend to inhibit the side of the head which normally would have developed first. Child and Watanabe (1935) and Watanabe (1935*b*) have presented data which indicate that the factors arising at the posterior cut surface which prevent the dedifferentiation and activation of the head-forming cells at the anterior cut surface are transmitted from their posterior point of origin chiefly by way of the ventral nerve cords. In these experiments with an oblique-posterior half-section only one of the nerve cords was severed, although it is possible that occasionally the other nerve was injured because of the proximity of the cut.

A comparison of the data furnished by these experiments on the removal of a block of tissue from behind an oblique-anterior cut surface with the data presented by Child and Watanabe (1935) on the removal of a block of tissue posterior to a transverse cut surface is extremely interesting. These authors report that only 2 per cent of the reconstituting animals develop heads of a strongly inhibited type (1 teratophthalmic, and 1 teratophthalmic or slightly teratomorphic), although a large majority of the early heads are asymmetrical. The data with posterior-oblique half-section (Table I) show that 5–6 days after section (also the time of observation used by Child and Watanabe) 9 from a total of 105 animals have heads with only one eye and 34 have developed no eyes at all in the tissue of the anterior cut surface. By 12–13 days after section, 3 are still with a single eye while 12 of the 34 with strongly inhibited anterior reconstitution have failed completely to develop heads. An explanation for this difference in inhibition of heads when a posterior block of tissue has been removed is to be found in the gradient hypothesis. In the transverse sections the head arises in the middle of the anterior cut surface in response to the mediolateral gradient. The imposed posterior half-section is therefore effective in inhibiting only one side of the head, which results in its asymmetrical development but not complete inhibition. In oblique sections the situation is somewhat different, for here the head arises chiefly in response to an anteroposterior

gradient and is therefore not in the middle, but on the more anterior region, of an oblique-anterior cut surface. A parallel oblique half-section imposed behind the anterior region of an oblique-anterior cut surface may therefore affect the entire head, at least in its earlier development. This results in a greater inhibition of heads arising from an oblique cut surface with posterior half-section than from a transverse cut surface with posterior half-section.

When a block of tissue is removed in order to impose a posterior cut surface upon either an anterior-oblique or an anterior-transverse cut surface, the possibility that the tissue removal itself may present factors inhibiting growth at the anterior region must not be ignored. In other words, the removal of a block of tissue posterior to the site of the new head may cause a deficiency of material which conceivably could inhibit that side of the new head behind which the tissue was removed.

That nervous factors do play a very important part in the inhibition of heads in *Euplanaria* seems to be fairly certain from the data at hand; yet there can be no doubt that tissue relationships (i.e., size of piece) will determine the ultimate size of the eyes when they do appear. It must be remembered, however, that the eyes examined 5–6 days after section have by no means attained their ultimate size. Such early inhibition does not seem likely to be due to lack of tissue, as we know from a multitude of experiments that there is sufficient material to permit the growth of much larger eyespots than were examined. The complete inhibition of eyespots (at 5–6 days) in 34 out of 105 cases with the posterior block of tissue removed points to a nervous effect rather than a tissue deficiency. The complete inhibition of one eye in 9 cases points to similar conclusions.

The appearance of a secondary head in the region where the block of tissue was removed creates an interesting problem (Figs. 6, 7, 9, 10). These heads have arisen partly from a posterior cut surface and partly from an anterior cut surface. It is interesting to note that the side of the secondary head which does arise on the posterior cut surface commonly (though not always) shows signs of inhibition of the eye and auricle (Figs. 6, 9, 10). The inhibited side of the head arises from a region which must first undergo a re-

versal in polarity, a factor which may produce a delay in the side of the head it affects. Tissue deficiency may also in this case be a cause of inhibition.

It has been noted in the data (Table I), and particularly in the controls, that slight indications of a reversal of size of eyespots appear at 12–13 days after section. In 8 cases it was also noted that a third eye had appeared in the regulating head. The increase in size of the eyespot arising in the new tissue over its fellow suggests that the first eyespot to appear is related to only a rudiment of the cephalic ganglion and is not at its final location. With the growth of the cephalic ganglion another eyespot locus may be determined. The enlargement and changes which occur perhaps determine either a larger or an additional eyespot field.

The data presented in this paper suggest that the work of Rand and Boyden (1913) on oblique sections may be interpreted in terms of a physiological differential along the anteroposterior axis. The reversal in eyespot size they describe when a longitudinal strip is removed from the outer side of the body suggests strongly that this operation injured to some extent the nerve cord on that side of the body and thereby produced an inhibition of the head-forming cells on the order of our experiments with the removal of a posterior block of tissue.

It has often been noted that oblique strips give rise to individuals which are much more asymmetrical in reconstitution than those which reconstitute when only a single oblique cut has been made. In the oblique strips the head appears on the extreme anterior region of the anterior cut surface, while the tail is confined to the more posterior area of the posterior cut surface. Both develop very asymmetrically. It seems to be clear that the reason for this pronounced asymmetry is because of two factors: (1) the anteroposterior differential in rate of head formation, and (2) the inhibitory influence of posterior section. In the oblique strips it appears that the only region of the anterior cut surface which can develop a head is confined to the most anterior part of the cut surface, which, presumably because of its higher physiological activity, is able to overcome the inhibitory influences which have been imposed by a parallel posterior cut surface. In this connection it may be mentioned that

experiments by many students in Professor Child's laboratory have shown that very short pieces with parallel anterior and posterior-oblique cut surfaces may yield headless individuals. In this case it is apparent that the posterior cut surface has completely inhibited the entire area of the anterior cut surface. The work on oblique strips also shows quite clearly that the head does not arise in response to a stimulation by the severed ventral nerve cords. If the nerve cords were the chief factors in the stimulation, the new head would surely arise near the middle of an oblique-anterior cut surface instead of at the more anterior region.

## SUMMARY

1. A new head arising at an anterior-oblique cut surface made at an angle of 60° from the transverse axis of the body in the anterior zooid of *Euplanaria dorotocephala* is asymmetrical in that the eyespot nearest the longer margin of the piece usually develops first.

2. When a parallel oblique-posterior half-section is made behind the more anterior region of the oblique-anterior cut surface, there is a tendency for the size of the eyespots on the new head to be reversed or for the new head to be partially or completely inhibited.

3. Regulation of asymmetrical heads at an anterior-oblique cut surface takes place very early in the reconstitutional process, and the normal bilateral symmetry of the species usually results.

4. Factors which may influence the symmetry of the developing head are: (1) the anteroposterior physiological differential and (2) factors originating at the posterior cut surface which inhibit the activation and dedifferentiation of the head-forming cells.

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# DEVELOPMENTAL ANALYSIS IN PLUMAGE. I. THE INDIVIDUAL FEATHER: METHODS

(Two plates and six figures)

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## I. Introduction

INTRINSIC properties of the individual feather (capacity for regeneration, mode of growth and differentiation, and reaction characteristics) make it a valuable instrument in the analysis of certain biological problems. The "field" characteristics of the plumage tracts are not less significant.

In the series of papers in this number of *Physiological Zoölogy* we extend analysis with particular reference to the relation of measurable characteristics in the feather to the sequence of embryonic events in the regenerating germ. Several phases of this analysis have appeared in preliminary accounts (Juhn and Fraps, 1934, I–IV).

In this first paper of the group we describe methods for preparation of material, apparatus and methods for measurement of pattern components in the definitive feather, and geometric procedures based upon the application of simple constructions to photographs of mounted feathers. The geometric methods and their significance rest upon relations described in the second paper.

The second paper ("II. Plumage Configurations and the Mechanism of Feather Development") describes certain pattern configurations and the mechanism of growth in the germ which we believe required to account for the properties of these configurations.

The establishment of these relations permits the definition of gradient and symmetry relations in the plumage tracts; each tract is treated as a field, and each follicle as a point therein. These points are characterized numerically in terms of absolute magnitudes (lengths, growth-rates, etc.) and by developmental ratios.

[1] This investigation was supported in part by funds from a grant by the Rockefeller Foundation to the University of Chicago in aid of biological research; one of the authors (R. M. F.) was the recipient of a grant-in-aid from the National Research Council.

In the third paper ("III. Field Functions in the Breast Tracts") we apply the methods described here and other measures to analysis of asymmetry and gradient relations as these are illustrated on transverse co-ordinates of the breast tracts.

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The geometric procedures described in this paper make possible the formulation of relations (e.g., barb frequencies) which are of considerable interest as phases of reaction thresholds in the different plumage tracts (see particularly Juhn and Gustavson, 1930). We shall present elsewhere the results of analyses of regenerations under the action of female hormone and thyroxin.

It need scarcely be emphasized that the phases of development recorded as measurable magnitudes or "frequencies" in the feather will often require transformation in terms of dynamic properties of the regenerating germ, e.g., growth-rates. This combination of physiological and geometric analysis is of particular value in defining the genetic complements of plumage, especially in connection with the field characteristics of the different plumage areas.

We have some data in this connection on hybrids of Barred Rock–Brown Leghorn crosses. These are of particular interest since the expression of the genic complement rests with some developmental property of the individual follicle which changes in intensity during the regenerative process and in successive regenerations (Juhn, 1933; see also Danforth, 1935).

## II. Preparation of Material

We consider here only magnitudes or frequencies which can be determined in the plane of the feather vane. These include the distribution of barbs on the shaft, length of barbs, and segments of barb and shaft defined by pigmentation or other configurations.

The most convenient orientation of the feather is with shaft straight, barbs at right angles to shaft or line of barb-shaft union. Feathers so mounted represent a system of natural co-ordinates in which shaft lengths are treated as abscissas, the barbs as ordinates. Thus, if Plate I is viewed with the shaft horizontal, apex of the feather to the left, the right vane-half is seen as a simple co-ordinate system. The left and right vane-halves of the feather shown in Plate

I are represented in text Figure 1 by lines drawn through the apexes of barbs.

A simple mounting-frame, shown in text Figure 2, is used in preparing feathers. The device consists of a board, *A*, with side members, *B* and *C*, serving as guides for the sliding member, *E*. At right angles to the line of motion of the board, *E*, is a guide bar, *D*, against which the brush or needle used in final alignment of the barbs is

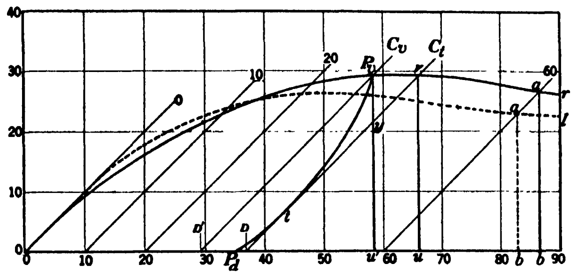


FIG. 1.—Marginal contours of the vane-halves of secondary major covert from a Brown Leghorn capon. Barbs (as *ab*, left and right vane-halves) lie at right angles to the line of barb-shaft union; the heavy curves pass through the apexes of barbs thus ordered. Abscissas represent millimeters from apex of the primary barbs; ordinates, millimeters from line of barb-shaft union. The diagonals, 0, 10, 20, and 60 represent loci of simultaneously uniform growth in the feather vane-halves; with barbs mounted at 90° to the line of barb-shaft union, the c-isochrone forms an angle of 45° with the same base line (axis of abscissas).

The curved line, $P_vP_d$, in left vane-half only, is an assumed locus of simultaneous pigmentation; maximum differentials involved in pigmentation are measured by the difference between c-isochrones $C_v$ and $C_t$ at their shaft intercepts.

moved at right angles to the shaft. The feather is applied to the surface of heavy illustration board or similar material; this board, or base, is held on the sliding member, *E*, with thumbtacks during the mounting operation. Feathers with large and stiff shafts are mounted on the type of base shown in text Figure 2. This base is made up of a lower block of illustration board, *F*, upon which are cemented two upper blocks, *G* and *H*. These blocks are in contact at or near one end; at the other they are separated sufficiently to accommodate the increased width of shaft from apex to base. For feathers with relatively slight shafts—e.g., saddle and breast feathers—the groove, *K–L*, is cut or impressed into a single piece of board.

In mounting the feather, the shaft is first set with hot paraffin into the groove, *K–L*. No particular attempt is made to straighten the barbs in this operation; the shaft is simply pressed into the groove and held down until the paraffin, applied with a brush, has cooled and hardened. The barbs are then pulled out at approximately right angles to the shaft with a brush carrying hot paraffin; we begin at the base and work to the apex of the feather. In the final operation the barbs are brushed out as accurately as possible into the right-angle position, and surplus paraffin is removed at the same time. The bar, *D*, serves in this operation to guide the brush as the feather is moved from base to apex under it.

A much more delicate final procedure is to bring the barbs to right angles with the shaft (or line of barb-shaft union) with a needle intermittently heated just sufficiently to melt the paraffin surrounding the barb and allow its alignment. The relatively small amount of heat carried by the needle is quickly dissipated, and the barb is "frozen" into position almost at once. However time-consuming, this technique gives us highly accurate mounts; these are particularly valuable for analysis of configurations directly from photographs. The feather represented in Plate I is an example of this technique.[2]

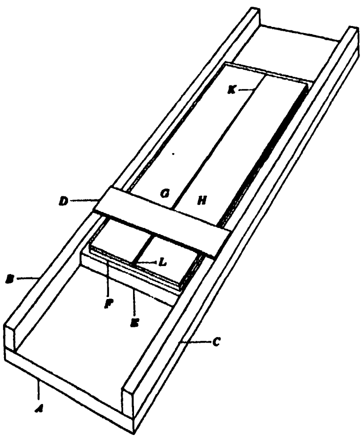


FIG. 2.—Mounting-frame used in preparation of feathers with barbs at right angles to the shaft; description in text.

Feathers thus mounted in paraffin are easily damaged; they can be given considerable protection by application of a film of shellac, collodion, or Duco. The protective film is applied with a spray.

In the permanent mounts the shaft of the feather has, of course, been straightened. Curvatures of shaft are very probably of im-

[2] We wish to express here our appreciation of the accuracy displayed in the feather mounts prepared by Miss Annelise Gerloff, some of which are reproduced in the three papers of this series.

portance, both as measures of differential rates (e.g., growth-rates) and as corrections to be applied to other data, as, for example, the exact angular relation of fault bars and shaft. We determine the curvature of shaft before mounting the feather by adjusting a flexible curve to the shaft in the required planes, and transfer these curves to paper.

Barbs are generally mounted at right angles to the *axis* of the shaft; in some feathers (e.g., the primaries) the shaft increases rapidly in diameter near the base. If direct measurements are made of barb and shaft lengths, no error is introduced, inasmuch as our base line is the junction of barb and shaft; in photographic analysis this actual base line—not the central axis of the shaft—must be determined and the degree of error so introduced taken into account unless barbs are mounted at right angles with it.

## III. Mechanical Measurement of Magnitudes in the Feather

The significant configurations in the definitive feather are determined in terms of lengths or distances. The magnitudes which must be measured vary from fractions of a millimeter—e.g., distances between barbs—to well over 40 mm. for total barb lengths. Also, a relatively great range of motion is required since, as a rule, we must proceed continuously from apex to base of the feather, as in measurement of barb distribution. The machine described here satisfactorily meets these requirements.

Photographs of the apparatus are shown in Plates IIA and IIB. The fixed microscope carries cross-hairs against which all settings are made. Range of motion of the stage is 220 mm., both in the line of motion of the gauges and at right angles to this axis. Measurements of stage displacement are made with two gauges, which may be used singly or in combination: a vernier scale reading to 0.02 mm. and a micrometer reading directly to 0.01 mm. The vernier operates over the full range of stage motion; the micrometer has a range of 25 mm. and is used primarily for measurement of barbule distribution on barbs, etc.

The mounted feather is fixed to the stage with thumbtacks (or with clips, not shown). The feather in the positions shown in Plates

IIA and IIB is oriented for measurement of distances along the shaft, e.g., distances between successive barbs. Exact alignment of the shaft with the axis of motion of the gauges is obtained by rotation of the stage, adjustment *T*, and transverse displacement of the stage, adjustment *P*.

If the micrometer is locked (*M*, Pl. IIB), any point on the shaft of the feather is brought under the cross-hairs by turning the vernier adjustment, *K*. Similarly, with the vernier locked on its scale (*V*, Pl. IIB), the stage is moved by turning *L* and reading positions on the micrometer scale.

The range of the micrometer may be extended over the full length of stage motion by resetting to zero. The vernier is used to shift the stage to the end-point of the previous series of micrometer readings. Again, the vernier may be used to move the shaft to any selected point in its length, and the distance between several barbs on either side of the chosen points of reference measured off with the micrometer. This procedure is convenient and rapid for determination of barb distribution.

Measurements along the axes of barbs are made with the stage at right angles to the position just described, i.e., with the barb axis in the line of motion of the gauges.

The transverse adjustment, *P*, is for exact location of an axis. It is not sufficiently rapid for many purposes, as, for example, in the location of barbs at intervals along the shaft in order to determine the approximate contour of the feather. The required rapid shift in the transverse direction is accomplished by lifting the lever, *S*, moving the stage to approximately the point desired, dropping the lever, and making the exact adjustment with *P*.

Many pattern configurations—e.g., the marginal contour just mentioned—may be located by still another procedure. The position-scale, *u*, calibrated in 0.5 mm., is parallel to, but independent of, the line of motion in the transverse sense. A pointer, *v*, moves with the transverse carriage. With the shaft of the feather in the line of transverse motion, points along its length are set off against the linear scale. The mean barb length corresponding to these positions on the shaft is then determined in the usual manner.

The apparatus described here has proved particularly convenient

in virtue of its range of stage motions, rapidity of operation, and the fact that readings are made directly from scales. Inasmuch as it may be useful in other applications, a brief note on its construction is appended (p. 316).

## IV. Geometric Analysis of Regenerated Feathers

We define the collar-isochrone,[3] or "c-isochrone," as the locus of simultaneously uniform cell division in axial sense. The barb-shaft co-ordinates of the c-isochrone are constant in the following terms: *The locus of simultaneously uniform cell division in axial sense is defined in the regenerated feather by the locus of points equally distant on barbs and the shaft from points of union of barbs with the shaft.* Let $P_v u'$, text Figure 1, be any barb crossed by the c-isochrone $VD$; the c-isochrone is completely defined by the relation $Du' = u'y$. If both vane-halves are plotted with barbs at right angles to the line of barb-shaft union (abscissa of text Fig. 1), $D$ is the common point of incidence of opposed c-isochrones and the angle $VDu' = 45°$.

In terms of growth, the c-isochrone represents a locus of points surrounding the base of the germ in the region of primary cell division. The projection of the originally annular locus of cell division in the germ upon corresponding elements in the regenerated feather takes the form of diagonals (at 45° to the shaft if barbs are mounted at 90° to the shaft), in consequence of uniform axial increments in barbs *and shaft;* the important c-isochrone constant is the identity of barb and shaft co-ordinates with respect to the point of union of any barb with the shaft, and not a given angular formation.

We are concerned here solely with magnitudes and relations which can be defined on the assumption that the c-isochrone represents in fact the locus of simultaneously equivalent growth by cell division at the base of the regenerating germ. The geometric procedures are straightforward; the exact interpretation of data thus obtained must always return, however, to actual rather than inferred developmental and growth relations in the germ. We call attention to the

[3] The term "isochrone" is due to Hardesty (1933), who used it to denote the locus of simultaneous pigmentation; for further description and distinctions, see Fraps and Juhn (1936*a*).

following points particularly (these are dealt with in the second paper of this series and are noted only in summary here):

1. We assume here that the c-isochrone is defined by exactly equal barb and shaft co-ordinates. On theoretical grounds it is probable that small but highly significant growth differentials (by cell division) characterize the locus of the c-isochrone in the germ. The exact co-ordinates of the c-isochrone in the regenerated feather may thus be different (however slightly) from the ideal construction here employed.

2. All levels represented by points of incidence of the c-isochrone on barbs and shaft are assumed to represent also simultaneously equivalent loci of cell division in the germ. It does not follow, however, that a c-isochrone (or c-isochrone parallel) in the collar defines the definitive structures (barb and shaft sections) of the same locus in the regenerated feather. The c-isochrone defines *loci* of cell division, and these loci may become levels of one or another element without change in co-ordinates of the c-isochrone itself.

3. The "ideal" uniform co-ordinates of the c-isochrone are in no sense evidence that differentials in reaction do not characterize different ventro-dorsal positions in the collar. On the contrary, any simultaneously imposed reaction which is not parallel to the c-isochrone in the regenerated feather can only be understood to represent some order of differential with respect to the c-isochrone as base.

Hardesty (1933) has described methods for analysis of the regenerated guinea-fowl feather.[4] Her procedures are based on the assumption that the locus of simultaneous pigmentation in the vane is approximately parallel with the locus of growth by cell division. This is apparently true as a limiting case, but in general there is considerable difference between the two loci (Fraps and Juhn, 1936*a*). The two methods give results which differ in proportion to the differences in configurations of the base lines used by Hardesty and by ourselves.

On one point particularly, our methods and results differ from

[4] We are, of course, concerned here with general methods. The application of relations in the regenerated feather to determination of antecedent relations in the germ is implicit in much of the analysis of Lillie and Juhn (1932), Montalenti (1934), and others. The suggestion of Lillie and Juhn that barb growth might be determined by measurement of distances between spaced female bars in male breast feathers is of particular interest in this connection.

those described by Hardesty: the base line from which our analysis proceeds is defined in terms of barb and shaft co-ordinates which are applied directly to the vane of the feather. Removal of barbs and spacing them at uniform intervals, with barb apexes forming a base line, introduces curvatures and therefore differentials of variable order, where none exist according to our findings (fault bars, described in second paper of this series). The methods of Hardesty yield results at variance with ours in these instances quite apart from differences due to application of differing base isochrones.

Hardesty's pigmentation isochrone defines a perfectly tangible set of relations, although we do not believe that these relations can be referred directly to the zone of growth by cell division. The p-isochrone is particularly valuable in defining the space order of ventro-dorsal differentials involved in pigmentation reactions, a point touched upon briefly below (see also Fraps and Juhn, 1936*a*).

### A. CONSTRUCTIONS ON PHOTOGRAPHS

Since the c-isochrone is a uniform construction, symmetrical in opposite vane-halves at all transverse shaft levels, any number of them may be drawn into photographs of feathers with barbs mounted at a constant angle with the (straightened) shaft. The feather reproduced in Plate I will be taken to illustrate procedures. In practice, all constructions are made with a fine stencil; relatively heavy lines and points on the photograph are required for reproduction.

Transverse shaft levels are referred to the apexes of the primary barbs as origin; shaft distances are always taken from this point as o unless otherwise noted. The distance from apexes of the primary barbs to the first mounted barb in each vane-half, and the spacing of the unmounted barbs in these lengths, are determined under the microscope.

Having determined the point *A* (o of graphs), a line is constructed from *A* through the apexes of all barbs mounted at right angles to the shaft; this continuous line will be called the "marginal contour," and it is treated as the margin of the vane-halves without further reference to individual barb apexes.

We now lay off at right angles to the central axis of the shaft, *AB*,

a series of transverse lines across the shaft. These transversals are laid off at known distances (with due reference to the usual enlargement of the feather in reproduction) from $A$, and represent *at the shaft* the axial co-ordinate of the regenerating germ to which all other data are referred. It is convenient in constructing the transversals to clamp the photograph with a straightedge parallel to the central axis of the shaft, $AB$ of Plate I, and draw in the lines with a right-angle triangle.

The point at which the transversal intersects the lines of barb-shaft union in each vane-half will be called the "shaft intercept." The straightedge is next aligned with the line of barb-shaft union, $AU$ of Plate I; and with a 45° triangle set at successive shaft intercepts we locate points of intersection with the marginal contour of the corresponding vane-half. These points are identified by drawing a short line across the contour at each intersection; they will be referred to as "marginal intercepts." The straight line from shaft intercept of any transversal to the corresponding contour intercept is, of course, a c-isochrone.

In general (excepting always certain apical formations) the error introduced by deviations of barbs from exact right angularity with the line of barb-shaft union is small in location of the marginal contour, relatively great in calculation of cumulative barb numbers. These latter errors are minimized by drawing from the shaft short lines lying on the perpendiculars (to line of barb-shaft union, $AU$, Pl. I) of successive marginal intercepts. In all relations involving barb numbers, the term "marginal intercept" refers to numbers *at the shaft* as determined by these perpendiculars.

We shall refer to c-isochrones by number, the number in any given case representing the distance in millimeters from apexes of the primary barbs to the shaft intercept of that c-isochrone. The o-isochrone is always the c-isochrone constructed from the apexes of the primary barbs, when these lie in the axis of the shaft. By *opposed* c-isochrones we shall denote c-isochrones in opposite vane-halves with identical shaft intercepts.

### B. MAGNITUDES AND RELATIONS DEFINED BY C-ISOCHRONES

The principal data obtained from the regenerated feather by c-isochrone constructions can best be illustrated by taking an example

(Pl. I), together with tables and graphs. All graphs presented in this connection are based upon c-isochrones at 1-mm. intervals from apexes of the primary barbs (0) to 10 mm., at 2-mm. intervals from 10 to 20 mm., and at 4-mm. intervals from 20 to 60 mm. The tables include only sufficient data to illustrate procedures.

### 1. CUMULATIVE BARB NUMBERS

The *marginal barb number*, $N_m$, is defined as the number of barb apexes lying between the apex of the primary barb (No. 1 in each vane-half) and the contour intercept of any c-isochrone. The *shaft barb number*, $N_s$, is defined as the number of barb bases lying between shaft intercepts of isochrone 0 and any c-isochrone at lower levels. Marginal or shaft barb numbers taken from the 0-isochrone through a series of c-isochrones are described as "cumulative" barb numbers.

Table I (cols. 2–5) gives cumulative barb numbers for c-isochrones located at various distances in millimeters (col. 1) from the 0-isochrone for the feather reproduced in Plate I. The complete data are given in Figure 3, curves $N_m$ representing marginal numbers, curves $N_s$ shaft numbers (ordinates), at successive axial levels (abscissas in millimeters).

Table I carries entries at 0 mm. (col. 1) for marginal barb numbers (cols. 2 and 3). These numbers represent the complement of barb loci which formed simultaneously in opposite limbs; the ventral-most barb in each collar complement defines the marginal intercept of the c-isochrone 0 (Fraps and Juhn, 1936*a*).

In all cases, marginal barb numbers represent the ventral-most limit of growth in the germ (with due regard to the notations above); and similarly, shaft barb numbers represent the dorsalmost limit of growth in the collar defined by a given c-isochrone.

### 2. BARBS ON C-ISOCHRONES

The number of barbs lying along a c-isochrone, $N_c$, is obtained by subtracting the shaft barb number from the marginal barb number defined by the same c-isochrone ($N_m - N_s = N_c$). For the c-isochrones entered into Table I (col. 1), the number of barbs on c-isochrones is given in column 6 for the left vane-half, in column 7 for the right vane-half.

Curves $N_c$ of Figure 3 represent the number of barbs on c-isochrones from apex of the primary barbs to the 60-mm. shaft level, left ($l$) and right ($r$) vane-halves. The ordinates of these curves are of course equal to the difference in ordinates of curves $N_m$ and $N_s$ (for each vane-half) at all levels from the apex of the feather (abscissas).

The barbs on a given c-isochrone are all simultaneously "represented" in the regenerating germ by cells in process of cell division.

TABLE I

PRIMARY C-ISOCHRONE DATA

| d | Cumulative Barb Numbers | | | | Barbs on Isochrones | | Barb Lengths | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Margin | | Shaft | | | | Margin | | Shaft | |
| | l | r | l | r | l | r | l | r | l | r |
| (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) | (9) | (10) | (11) |
| 0 | 25.0 | 9.0 | 0 | 0 | 25.0 | 9.0 | 12.0 | 5.0 | ...... | ...... |
| 1 | 36.8 | 24.0 | 1.0 | 1.0 | 35.8 | 23.0 | 16.6 | 11.1 | 1.0 | 1.0 |
| 2 | 43.5 | 31.4 | 2.2 | 2.2 | 41.3 | 29.2 | 18.2 | 13.3 | 2.0 | 2.0 |
| 3 | 49.2 | 37.8 | 4.0 | 3.9 | 45.2 | 33.9 | 19.5 | 15.1 | 3.0 | 3.0 |
| 10 | 76.8 | 71.2 | 19.7 | 18.9 | 57.1 | 52.3 | 24.2 | 23.2 | 10.0 | 9.4 |
| 12 | 83.3 | 79.2 | 24.5 | 23.4 | 58.8 | 55.8 | 25.0 | 24.8 | 12.0 | 10.8 |
| 14 | 89.6 | 86.1 | 29.2 | 27.8 | 60.4 | 58.3 | 25.3 | 26.0 | 13.8 | 12.2 |
| 20 | 106.3 | 104.3 | 43.1 | 41.1 | 63.2 | 63.2 | 26.5 | 28.0 | 17.8 | 16.0 |
| 24 | 116.7 | 115.4 | 52.4 | 49.9 | 64.3 | 65.5 | 26.6 | 28.9 | 20.0 | 18.6 |
| 28 | 126.4 | 125.9 | 61.9 | 58.7 | 64.5 | 67.2 | 26.4 | 29.3 | 21.9 | 20.5 |

In terms of development, then, the number of barbs on a c-isochrone may be taken as a measure of the number of barb primordia "represented" in the collar, wholly apart from the actual number of definitive primordia present in the collar at the same moment.

### 3. BARB FREQUENCIES

The *marginal barb frequency*, $f_m$, is defined as the number of barb apexes lying between two c-isochrones 1 mm. apart at their shaft intercepts. Similarly, the *shaft barb frequency*, $f_s$, is defined as the number of barb bases lying between two c-isochrones 1 mm. apart at their shaft intercepts.

Barb frequencies based upon the data of Table I are given in Table II. Column 1 of Table II gives the *isologue* number corresponding to intervals represented between successive c-isochrones (isologue 1 is the interval between c-isochrones 0 and 1; isologue 2 is the interval between c-isochrones 1 and 2, etc.). Column 2 ($s$) records the distance in millimeters between the c-isochrones bounding the isologues of column 1.

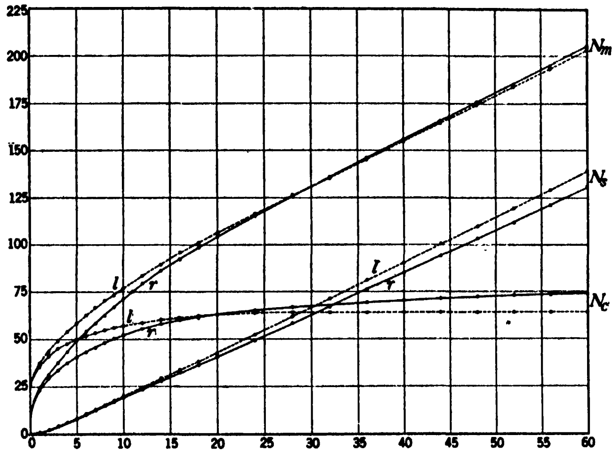


FIG. 3.—Curves representing cumulative barb numbers and number of barbs cut by successive c-isochrones, major covert No. 13, Brown Leghorn capon (Pl. I; contours shown in text Fig. 1). Abscissas, millimeters from apex of primary barbs; ordinates, barb numbers. Broken lines ($l$), left vane-half; solid lines ($r$), right vane-half. Curves $N_m$, cumulative barb numbers at the margin; $N_s$, cumulative barb numbers at the shaft; $N_c$, numbers of barbs cut by successive c-isochrones.

If c-isochrones are spaced 1 mm. apart, the marginal barb frequency is obtained directly by subtracting the marginal barb number defined by a given c-isochrone from the marginal barb number of the succeeding c-isochrone (col. 2 of Table I, $36.8-25.0=11.8$; entered under col. 5 of Table II). Shaft barb frequencies are obtained similarly from shaft barb numbers (cols. 9 and 10, Table II).

If c-isochrones are spaced at intervals other than 1 mm., marginal barb frequencies are calculated according to the relation

$$f_m = \frac{n_m}{s},$$

where $n_m$ is the number of barb apexes between successive c-isochrones and $s$ is the distance in millimeters between successive c-isochrones at their shaft intercepts. Shaft barb frequencies are calculated similarly, except that $n_s$, the number of barb bases between successive c-isochrones, replaces $n_m$, and we have

$$f_s = \frac{n_s}{s} .$$

Barb frequency curves for the secondary covert reproduced in Plate I are shown in text Figure 4 ($f_m$, marginal barb frequencies; $f_s$,

TABLE II

BARB FREQUENCIES

| d | s | $n_m$ | | $f_m$ | | $n_s$ | | $f_s$ | |
|---|---|---|---|---|---|---|---|---|---|
| | | *l* | *r* | *l* | *r* | *l* | *r* | *l* | *r* |
| (1) | (2) | (3) | (4) | (5) | (6) | (7) | (8) | (9) | (10) |
| 1...... | 1 | ....... | ....... | 11.80 | 15.00 | ....... | ....... | 1.00 | 1.00 |
| 2...... | 1 | ....... | ....... | 6.70 | 7.40 | ....... | ....... | 1.20 | 1.20 |
| 3...... | 1 | ....... | ....... | 5.70 | 6.40 | ....... | ....... | 1.80 | 1.70 |
| 12...... | 2 | 6.5 | 8.0 | 3.25 | 4.0 | 4.8 | 4.5 | 2.40 | 2.25 |
| 14...... | 2 | 6 3 | 6.9 | 3.15 | 3.45 | 4.7 | 4.4 | 2.35 | 2.20 |
| 24...... | 4 | 10.4 | 11.1 | 2.60 | 2.78 | 9.3 | 8.8 | 2.33 | 2.2 |
| 28...... | 4 | 9.7 | 10.5 | 2.43 | 2.63 | 9.5 | 8.8 | 2.38 | 2.2 |

shaft barb frequencies; *l* and *r*, left and right vane-halves; abscissas, distances from c-isochrone 0). The curves are based upon the smooth curves, $N_m$ and $N_s$, of text Figure 3. This procedure tends to eliminate fortuitous fluctuations and to reduce errors in the original measurements, but obviously must be used with due regard for the nature of initial data. The circles and disks of text Figure 3 represent actual determinations.

## 4. RATE FUNCTIONS OF C-ISOCHRONES

Barb frequencies represent the actual numbers of "discrete events" which, *after the completion of regeneration*, have been effected per millimeter axial growth. Within the limitations of this definition, we may speak also of "rate functions," marginal and shaft. In order

not to confuse collar level and time of origin of the definitive barb primordium with c-isochrone determinations, we shall define the *marginal rate function* as the number of barb apexes determined per day *as ventral loci* with respect to c-isochrone constructions. The shaft rate function is correspondingly the rate at which the loci of union of barb bases with shaft are determined by c-isochrones.

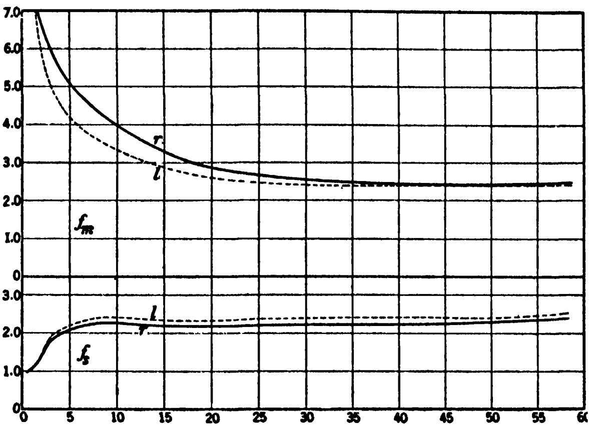


FIG. 4.—Marginal and shaft barb frequencies, major covert No. 13, Brown Leghorn capon; broken lines ($l$), left vane-half; solid lines ($r$), right vane-half. Abscissas, distances in millimeters from apexes of primary barbs; ordinates, number of barbs per millimeter at the margin ($f_m$) and at the shaft ($f_s$). Based upon the smooth curves of text Figure 3.

Knowing the axial growth of the germ,[5] $g$, in millimeters per day, the marginal rate function, $r_m$, is obtained from the marginal barb frequency according to the relation

$$r_m = gf_m \, .$$

The shaft rate function, $r_s$, is obtained similarly, except that the shaft barb frequency replaces the marginal barb frequency in the foregoing relation, and

$$r_s = gf_s \, .$$

[5] We refer to longitudinal growth of the germ, which, of course, is continuous for the shaft during the course of a regeneration, as the axial growth or (growth-rate) of the germ; tangential growth (or growth-rate), effecting the ventrodorsal motion of barb primordia, does not enter directly into the relations under discussion here (see Fraps and Juhn, 1936*a*).

In the limited but exact terms here defined, the rate functions of c-isochrones are a measure of one phase of differentiation in the regenerating germ; the same is, of course, true of barb frequencies. The subject requires further examination in terms particularly of the relation between axial and tangential growth motions (Fraps and Juhn, 1936*a*).

### 5. GROWTH-RATES IN INDIVIDUAL BARBS

The lengths of all barb segments lying between two c-isochrones are equal among themselves, and they are equal also to the length of shaft lying between the same c-isochrones. On the grounds of the usual assumptions concerning growth, we interpret this to mean that the rate of axial growth by cell division is simultaneously uniform in a basal annular locus of cell division (Fraps and Juhn, 1936*a*). Stated differently, points of c-isochrone incidence on the shaft and all barbs are levels of simultaneously uniform axial growth-rate.

The rate of growth of the shaft can be measured during the course of regeneration. These measurements involve growth by cell division and growth due to increase in size of cells following cell division. It is necessary to consider this second phase of growth a constant function (in total increment effected, not in rate) of growth by cell division, whence it follows that axial growth by cell division within the collar (uniform on a given c-isochrone), remains a constant function of the measured total growth of the shaft.

### 6. TERM OF BARB GROWTH

If the growth curve is known for a regenerated feather, the term of barb growth is determined by locating c-isochrones through the apex and base of the individual barb; the term of growth is identical with the time required for growth of the shaft segment so defined.

If the axial rate of growth of the shaft can be taken as constant through the term of a barb's growth, the term of barb growth is obtained by the relation

$$t=\frac{l}{g},$$

where $t$ is the time in days required for growth of the barb, $l$ is the length of the barb in millimeters, and $g$ is the axial growth of the shaft in millimeters per day through the interval of barb growth.

### 7. BARB LENGTHS

Curves for barb lengths at completion of barb growth, barbs ranged at right angles with the shaft, are identical with marginal contours as these have been defined previously (text Fig. 1: ordinates, barb lengths in millimeters; abscissas, distances on shaft in millimeters).

Since the c-isochrone is symmetrical at shaft intercepts, barb lengths in opposite vane-halves at any c-isochrone shaft intercept represent lengths of barbs simultaneously completed. If these opposed barbs are equal in length, their apical (marginal) c-isochrone loci were also simultaneously determined; if different in length, the difference can be reduced to a time interval separating origin of the marginal loci in opposite vane-halves.

The marginal intercepts of opposed c-isochrones represent loci of simultaneous origin (apexes *a*, right and left vane-halves, c-isochrone 60, text Fig. 1). The distances of these points from the shaft (ordinates) represent therefore the lengths attained by simultaneously defined barb apexes at completion of barb growth. The complete curves for axial displacement of marginal loci of simultaneous determination are given in text Figure 5 for the same feather represented in text Figure 1 (see also Table I). Note particularly the striking differentials between opposite vane-halves through first 10 mm. of feather growth.

An obvious significance of barb lengths in either of these instances is that they represent time intervals of axial growth of the individual barb in the germ (see secs. 5 and 6 above); other relations into which we cannot enter here involve rates of tangential displacement of the defined loci.

### 8. GEOMETRIC VARIABLES IN PIGMENTATION FORMATIONS

Pigmentation formations in the germ may be effected simultaneously at all ventro-dorsal positions in the collar (Lillie and Juhn, 1932; Hardesty, 1933). We shall refer to such simultaneous formations as *p-isochrones*. We consider here only relations which are defined by application of the c-isochrone construction to these simultaneously determined configurations.

Let $P_vP_d$, text Figure 1, represent a p-isochrone in the left vane-

half of the feather. If any p-isochrone is not parallel to the c-isochrone; c-isochrones constructed through various loci of the p-isochrone will have different shaft intercepts. Thus, in text Figure 1, a c-isochrone drawn through $P_v$ (ventral-most limit of pigmentation in the germ) will intersect the shaft at $D'$; similarly, a c-isochrone, $C_t$, drawn through $t$, the level of pigmentation nearest the base of the germ, will intersect the shaft at $D$. The two points, $D$ and $D'$, are separated by a distance which is equal to $P_v y$, and determine therefore the maximum differential in level of pigmentation for this

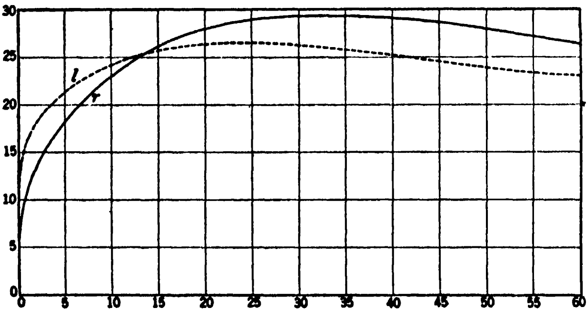


FIG. 5.—Lengths of barbs in left ($l$) and right ($r$) vane-halves at corresponding marginal intercepts of c-isochrones; major covert No. 13, Brown Leghorn capon. Abscissas, distances in millimeters from apexes of primary barbs; ordinates, barb lengths in millimeters.

particular formation. We may proceed similarly with c-isochrones through $P_d$, or with c-isochrones drawn through any selected order of loci lying along the p-isochrone.

The difference between cumulative barb numbers at the margin of c-isochrones $C_t$ and $C_v$ determines the least number of barb loci represented in the germ for this particular configuration (Fraps and Juhn, 1936*a*).

The significance of the measures derived by c-isochrone constructions to p-isochrone configurations is that the variable configuration of the p-isochrone can be reduced to quantitative (if relative) indexes. Several of the more general relations and applications may be touched on briefly here.

Pigmentation isochrones generally show an increasing deflection from the c-isochrone with increasing distance from the apex of the

feather. This increasing deflection can be reduced to numerical relations (as the distance, $P_vy$, or differences in marginal barb numbers).

Feathers of differing types (breast, wings, etc.) show characteristic differences in deflection of p-isochrones from c-isochrones; these differences are of particular interest in connection with their dependence upon physiological properties of the regenerating germ (Lillie and Juhn, 1932).

In a perfectly symmetrical feather, p-isochrones in opposite vane-halves are symmetrical by c-isochrone measures. If the p-isochrones are asymmetrical, the degree of asymmetry under differing conditions, at different axial levels of the same feather, and in different feathers, is determined in comparable and numerical terms by c-isochrone constructions. The procedure rests upon the determination of shaft intercepts of c-isochrones through *homologous* points on the p-isochrone locus (as the point $P_v$, text Fig. 1).

If we know the form of the p-isochrone at a given level of a particular feather, pigmentation configurations departing from the p-isochrone can be reduced to at least relative measures of the time differential involved in the germinal reaction by application of c-isochrones.

## V. Relative Ratios in the Analysis of Asymmetries

We are justified in speaking of the "asymmetry" of an individual feather only with respect to exactly defined magnitudes or relations in opposite vane-halves. The feather used to illustrate c-isochrone procedures (Pl. I) is, for example, obviously asymmetrical by every measure considered in the foregoing section (text Figs. 1, 3–5). But a satisfactory statement of asymmetry requires much more than this; it requires, first, a more compact treatment of the data; and, secondly, a dissociation of "independent" and "dependent" variables entering "asymmetrically" into the developmental process.

We are concerned here, as one phase of methods, with a simple relation which describes cumulative barb numbers in opposite vane-halves of many, if not all, feathers from certain tracts. The generality of the relation and its significance in development must be the subject of further investigation.

If we let $y$ represent the marginal barb numbers on successive

c-isochrones in one vane-half, $x$ the marginal numbers on corresponding c-isochrones in the opposite vane-half (curves $N_m$ of text Fig. 3), the relation between the two magnitudes is

$$y=bx+a\ ,$$

where $b$ and $a$ are constants over the range for which the relation obtains. The constant $b$ represents the ratio of cumulative barb numbers in opposite vane-halves on successive c-isochrones; if $b=1$, marginal barb numbers increase absolutely by identical increments. The value of $a$ in the equation is the value which $y$ assumes when $x$ is set equal to zero.

A similar relation is found to hold also between shaft barb numbers determined by opposed c-isochrones (curves $N_s$ of text Fig. 3 represent the initial data).

The equation $y=bx+a$ is, of course, the equation for a straight line on arithmetic co-ordinates. Wherever cumulative barb numbers (margin or shaft) are related according to this equation, numbers in one vane-half plotted against corresponding numbers in the opposite vane-half should fall along a straight line; the slope of this line is defined by $b$; and the $y$-intercept of the line is equal to $a$.

Text Figure 6 represents the cumulative barb numbers of the feather reproduced in Plate I treated as relative ratios. The relative marginal ratios (*ab* and *bc* of text Fig. 6) are based upon the actual data (not the smoothed curves) represented along curves $N_m$ of text Figure 1; the shaft ratios are based likewise on the actual c-isochrone determinations.

The relative ratio of marginal numbers falls into two well-defined phases. For the first phase, *ab* of text Figure 6, $y=1.20x-20.6$; for the second phase, *bc* of text Figure 6, $y=1.05x-7.3$. The relative rate of increase in marginal barb numbers is thus higher in the right vane-half for both phases (compare the curves of text Fig. 3).

The first plotted point, *a* of text Figure 6, represents the marginal intercepts of c-isochrone 0 (see p. 303), and is therefore referable to the number of barbs simultaneously determined in opposite vane-halves. In all cases where $b$ becomes a constant from this point, as through the phase, *ab*, text Figure 6, we may consider the simul-

taneously determined values of c-isochrone o ($y=9$, $x=25$, text Fig. 6) to be the proper origin of the curves, and the constant, $a$, then has no direct significance. The grounds for this treatment (assuming it to hold generally) are that serial formation of barb loci measured by the marginal numbers can begin only after formation of the simultaneous initial complements, and it is only these serial formations which can be treated as ratios.

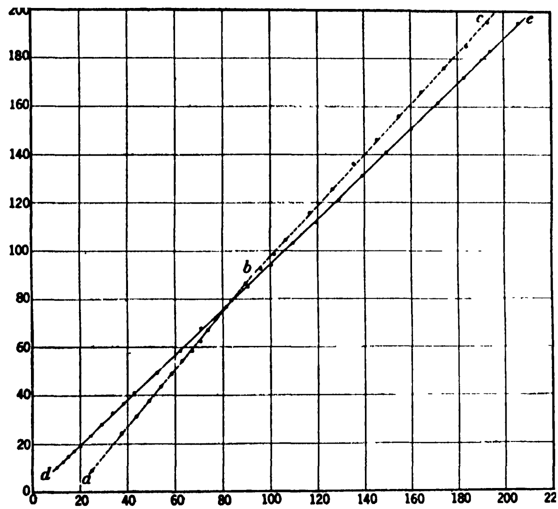


FIG. 6.—Relative ratios in increments of barb numbers in opposite vane-halves, major covert No. 13, Brown Leghorn capon (Pl. I). Abscissas, barb numbers in left vane-half; ordinates, barb numbers in right vane-half. Marginal numbers (circles) lie along the broken lines; for the first phase ($a$ to $b$), $y=1.198x-20.6$; for the second phase ($b$ to $c$), $y=1.053x-7.3$. Shaft numbers lie along the solid line ($d$ to $e$); $y=0.94x+0.5$.

Barb numbers for shaft intercepts of successive c-isochrones are plotted also in text Figure 6 (along the solid line, *de*; $y$-co-ordinates, right side; $x$-co-ordinates, left side). Over the range shown, $y=0.94x+0.5$. Theoretically, it may be supposed that $a$ should equal o for shaft numbers, since barb numbers at the shaft must begin with the common base of the primary barbs; this point requires further and exact analysis.

If marginal and shaft barb numbers in opposite vane-halves increase by constant ratios, it follows also that barb numbers lying

along successive c-isochrones in opposite vane-halves change (increase or decrease) by a constant ratio. This relation, and others of a less obvious character, will be treated in a more extended analysis.

We have found that the relation $y=bx+a$ holds generally in describing the relative barb ratios (marginal, shaft, and "collar") in opposite vane-halves of contour feathers, secondary coverts, and possibly tail feathers of the Brown Leghorn capon. Certain primary flights are not apparently subject to the relation (nor to the heterogeny formula of Huxley, 1932). In at least one well-defined instance (hybrid: Barred Rock ♂, Brown Leghorn ♀), the genetic composition appears to impose periodic fluctuations asymmetrically in opposite vane-halves.

In certain instances where the formula holds, the values of the constant $b$ for at least marginal barb numbers are definitely related with other asymmetry "indexes" (Fraps and Juhn, 1936*b*). In other instances no such general relation is apparent, as, for example, in certain coverts which are highly asymmetrical in appearance (the "appearance" is referable to marked differences in lengths of barbs) but in which the relative ratios of both margin and shaft numbers are close to unity through the main vane-structure.[6]

Relative increments in cumulative barb numbers at margins and shafts must involve, in part at least, growth vectors (Fraps and Juhn, 1936*a*). Axial growth of the germ is largely additive or "accretionary" (Huxley, 1932); and it might be supposed that, following completion of the collar complements and serial formation of barb primordia at the ventral triangle, tangential growth is to be treated also as primarily accretionary. On these grounds it might be concluded that the formula $y=bx+a$ rests in part upon a constant differential growth relation in an "accretionary system," corresponding therefore to Huxley's (1932) expression $y=bx^k+a$ in the multiplicative system. But cumulative barb numbers involve also what

[6] Although we have determined the value of $b$ for a large number of individual feathers, its general significance probably cannot be known until we have much additional data on the order of change of asymmetry measures for complete series of feathers in different tracts—a point which is clearly emphasized also by those instances in which the relative ratios of barb numbers depart considerably from unity although the feathers are fairly symmetrical in appearance.

might be called "differentiation frequencies," and we are inclined to forego interpretation until more comprehensive results are in hand. It is also altogether possible that further analysis will show the simple relation described here to be a special and limited aspect of a more general and inclusive relation.

## VI. Summary

1. Methods are described for mounting individual feathers with barbs at right angles to the shaft (or line of barb-shaft union).

2. An apparatus is described for the rapid measurement of magnitudes in the vane of mounted feathers; construction of the apparatus is appended (p. 316).

3. The c-isochrone is defined in terms of growth characteristics in the regenerating germ (based upon Fraps and Juhn, 1936*a*), and its application to photographs of mounted feathers is described. Magnitudes and relations determined by the c-isochrone construction are defined, and their significance in terms of development is discussed briefly.

4. Relative ratios in barb numbers, as these are determined by the c-isochrone, are considered in connection with the formulation of asymmetries.

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## APPENDIX

### CONSTRUCTION OF THE MEASURING APPARATUS

The entire apparatus is mounted on an aluminum casting, webbed for rigidity. For linear stage motions we have made use of the carriage mechanism of computation machines. These consist of a truck, *b* (Pl. IIA), moving on ball bearings within the carriage frame, *a*. The frame of the lower carriage is bolted to the aluminum base. Its truck carries a heavy plate, *c*, to which is bolted (at right angles to *c*) a second plate, *d*; these plates are centered with respect to each other.

The upper or transverse carriage is identical with the lower; its frame is bolted to the transverse plate, *d*, and its truck carries a plate corresponding to *c* on the lower truck.

Rotation of the stage is accomplished through worm and gear. The gear, *e*, is centered in the upper truck plate, turning on a large ground bearing. A circular stage plate is bolted to the worm gear; stages proper—several types have been used—bolt directly to this plate. The worm for transmitting motion to the gear is fixed in the center of the shaft, *t*, which is supported by bearings at each end of the upper truck plate.

Change in position of the transverse carriage truck is effected by rotation of the screw, *p*, with consequent displacement of the riding half-nut, *r*. The half-nut is attached—by way of the hinged strip, *f* (Pl. IIB)—to the truck plate of the transverse carriage; it is held in position by the spring acting through the lever, *S*. If this lever is raised, the half-nut is lifted clear of the screw threads and the transverse truck may be shifted to any position.

The position-scale, *u*, is attached to the lower truck plate; the pointer, *v*, is fixed to the upper truck plate. The pointer is a strip of spring steel bearing a mark practically in the plane of the scale calibrations.

Relations of vernier scale, micrometer, and lower carriage truck are clear from Plate IIA; details are shown in Plate IIB. Axes of motion of the scales must, of course, be in exact alignment with the axis of motion of the lower truck.

The vernier scale is mounted on a pair of end-blocks above a heavy bar, *z*, which

bar is bolted to the base of the apparatus. A pinion at the end of the shaft carrying $K$, engages a rack, $j$, fixed to the bar carrying the vernier scale. Pinion and shaft are held in position by a guiding frame which serves also for displacement of the vernier on its scale. The frame consists of a pair of side plates, $w$ (and its opposite), spaced by the block, $x$, and bearing at their inner and lower corners the guides, $y$ (and an opposite); these guide bars engage corresponding grooves in the bar, $z$.

The block, $x$, is slotted across its upper face to receive the vernier block, $i$. The vernier, then, lies with a shoulder of the spacing block on either side of it; displacement of the block brings one or the other of these shoulders against the vernier, and so moves it along the scale. The spacing-block does not touch the scale, and points of contact with the vernier are convex and in the line of the central axis of its motion. Movement of the vernier is thus free of possible distortion either of the scale itself or of alignment of the vernier with respect to the scale.

The original jaws of vernier and micrometer frame are removed except for small brackets for their union ($q$, Pl. IIB). The micrometer is of the type designed for internal measurements, in which the spindle, $m$, does not revolve with the thimble, $L$. A connecting rod, $n$, ties the micrometer spindle to the bracket, $o$ (Pl. IIA), which bracket is in turn fixed with respect to the lower carriage truck.

Fairly heavy construction has been necessary throughout, largely in consequence of the ranges of stage motions required of the apparatus. There appear to be no appreciable strains on the gauges, however, nor is there any evidence of inertial drags even in rapid operation of the vernier over relatively great lengths. The micrometer has been repeatedly checked to within 0.002 mm. in return to a previously located point- -about the limit of estimation possible between 0.01-mm. calibrations.

We have made considerable use of the apparatus in measurement of relations in split germ preparations, a procedure which requires illumination by transmitted light. Due to space limitations, the usual microscopic equipment is impracticable. A light source adequate to our requirements was obtained via a simple "neon type" installation (Courtney and Schopp, 1934). The discharge tube—of about $\frac{3}{8}$-inch diameter—is bent back upon itself several times to form a series of lengths lying close together and in a plane. The unit is fitted into a shallow container which replaces the stage shown in Plate II; a ground glass plate fitting into the side walls of the container bridges the parallel sections of the illumination tube. A pair of clips hold the slide in place.

## EXPLANATION OF PLATES

All figures are photographs.

### PLATE I

Photograph of major covert over secondary No. 13 (counting from wing tip through primaries), showing c-isochrone constructions. Central axis of the shaft, $AB$; line of barb-shaft union, $AU$ (in right vane-half). Marginal contours drawn only where required for c-isochrone intercepts. The herringbone diagonals, 0, 10, 20, 60, represent c-isochrones in each vane-half at levels corresponding to distances in millimeters from apex of the feather indicated by these numbers. The shaft transversals defining these distances ($D=60$ mm. from apex) are drawn at right angles to the central axis of the shaft. If barbs are mounted at right angles to line of barb-shaft union, $AU$, all other constructions assume this line as base. The angle $VDU=45°$ and locates the point $V$ on the marginal contour. The point $u$ is determined by the perpendicular passing through $V$ from $AU$ as base; similarly, the point $a$ is located as the point of intersection with the marginal contour of a line from $D$ at right angles to $AU$. If a number of c-isochrones are drawn into the vane of a feather, points $a$ and $V$ are distinguished by the angle of intersection of short lines with the marginal contours (90° at $a$; 45° at $V$); points $D$ and $u$ are identified by short lines of differing lengths.

### PLATE II A

Photograph of the apparatus used in the measurement of distances in feathers. The feather on the stage is oriented for measurement of distances along the shaft, e.g., distances between barbs. For measurement of barb lengths, the stage is rotated until the shaft lies at right angles to the position shown. Relatively great lengths are measured with the vernier (the micrometer is locked) by rotation of $K$. For more accurate measurements, the micrometer $L$, is used (the vernier is locked). Range of stage motions of the apparatus is 220 mm. The vernier reads to 0.02 mm.; the micrometer, directly to 0.01 mm.

### PLATE II B

Photograph showing details in combination of the vernier and micrometer scales. The micrometer is locked with the setscrew, $M$; the vernier, with the setscrew, $V$. Description in text; details of construction in Appendix.

PLATE I

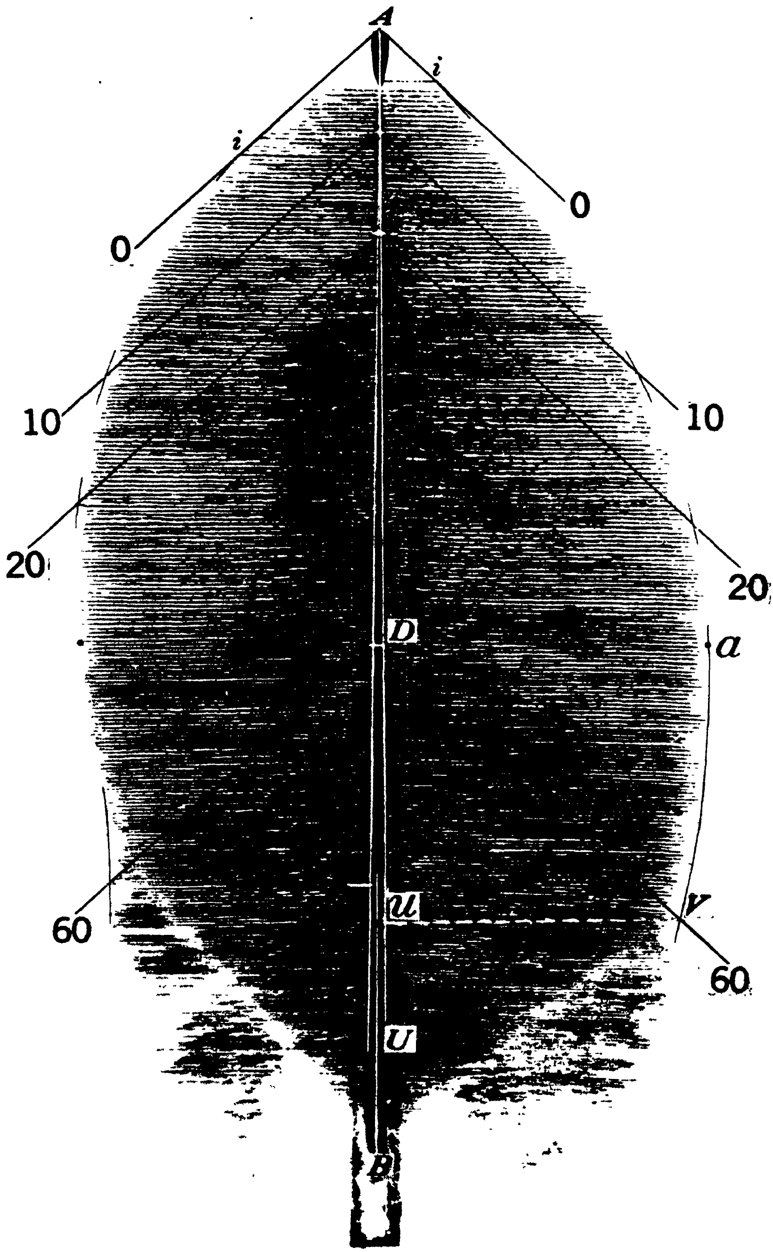


PLATE II A

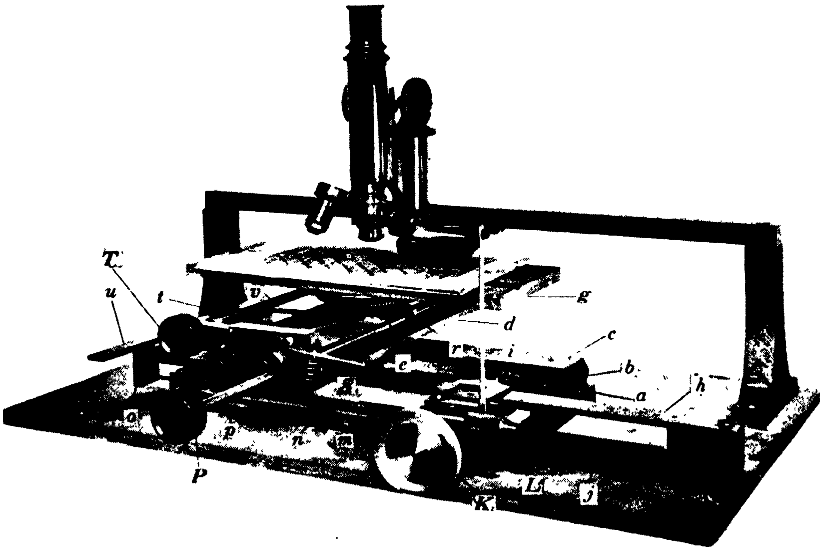
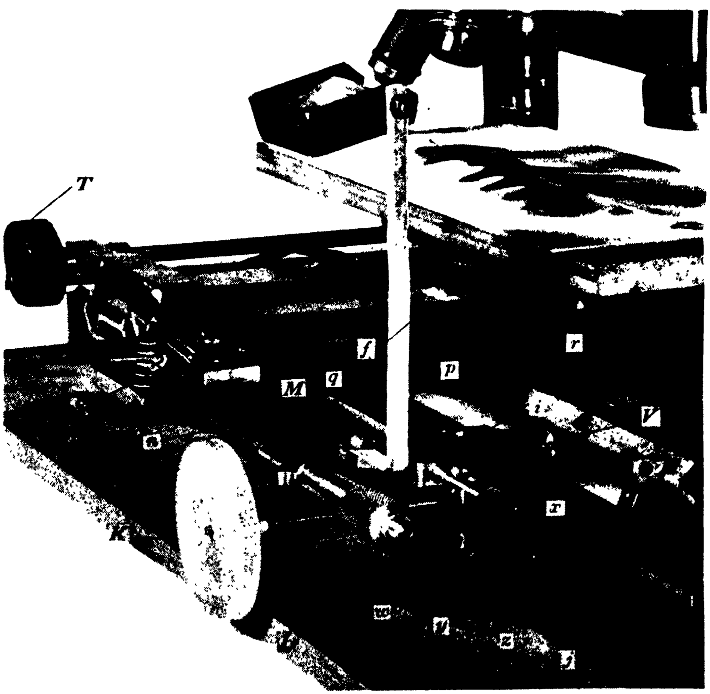



PLATE II B



# DEVELOPMENTAL ANALYSIS IN PLUMAGE. II. PLUMAGE CONFIGURATIONS AND THE MECHANISM OF FEATHER DEVELOPMENT

(Five plates and nineteen figures)

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## I. Introduction

IN THEIR analysis of growth-rate and pattern in feathers, Lillie and Juhn (1932) demonstrated that pigmentation patterns simultaneously induced in all the reactive elements of the germ come to lie, with completion of the regenerative process, in a line approximately at right angles to the shaft. Hardesty (1933) later showed that the series of simultaneously formed "spots" of the guinea-fowl feather could be treated as localized regions of reaction along lines approximately perpendicular to the shaft, although "the direction of these lines relative to the rhachis is subject to considerable variation."

On the basis of observations on the axial displacement of simultaneously induced pigmentation reactions following their appearance in the germ, Lillie and Juhn reached the conclusion that the axial rate of growth of the individual barb is highest at the apex and lowest at or near the base. The order of difference in apicobasal growth-rates was estimated to be several-fold. Hardesty obtained a similar curve for the individual barb growth-rate by analysis of pigmentation lines in completely regenerated feathers.

Juhn and Fraps (1934*a*) attempted to determine axial growth-rates of barbs by analysis of lines of simultaneous pigmentation and of fault-bar incidence (Riddle, 1908) in regenerated feathers. Taking into account results based only upon pigmentation configurations, growth-rate differentials estimated according to our procedures were

[1] This investigation was supported in part by funds from a grant by the Rockefeller Foundation to the University of Chicago in aid of biological research; one of the authors (R. M. F.) was the recipient of a grant-in-aid from the National Research Council.

a small fraction of the differentials obtained either by Lillie and Juhn or by Hardesty.

In the present paper we give results of further analysis of configurations in the completely regenerated feather. The simple and critical relation with which we are mainly concerned is that which defines the locus of axial growth by embryonic cell division in the regenerating germ. This locus of growth will be denoted the *c-isochrone,*[2] and it is completely defined in either vane-half of the regenerated feather as follows: *Points of incidence of the c-isochrone on the shaft and any barb are equidistant from the point of union of that barb with the shaft.* The c-isochrone is symmetrical with respect to a common transverse shaft level and is uniform in all feathers. It can be located, therefore, through the entire vane of the regenerated feather if a single point of incidence is known or given.

The experimentally determined geometric relations are shown in text Figure 1, which represents a portion of completely regenerated feather, base down, barbs removed below $u$ in left and right vane-halves. Let $C_d$ be the common point of incidence of the c-isochrones, $C_vC_d$, in opposite vane-halves. Considering only the left vane-half, let $u'w=b$, the barb co-ordinate, and $u'C_d=s$, the shaft co-ordinate. The general relation is that $b=s$ for any barb between the marginal limit, $C_v$, of the c-isochrone and the central limit, $C_d$. If the barbs are ranged parallel, as in text Figure 1, the c-isochrone is a straight line, $C_vC_d$, the base of the isosceles triangle, $C_vuC_d$.

The approximate form of the lines of simultaneous pigmentation described by Lillie and Juhn and by Hardesty is indicated by $P_vC_d$. The barb and shaft co-ordinates of the pigmentation configuration, $P_vC_d$, obviously cannot be identical with the co-ordinates of the c-isochrone, $C_vC_d$. In general, $b$ ($u'w''$ of text Fig. 1) is greater than $s$ ($u'C_d$ of text Fig. 1) for pigmentation isochrones if these are considered as approximately straight lines from margin to shaft.

The c-isochrone in the vane-halves of the regenerated feather represents the locus of simultaneous reaction through the zone of

[2] The term "isochrone" was first used by Hardesty (1933) with reference to the locus of simultaneous pigmentation in the germ. We have given the term a somewhat different meaning; the notation "c-isochrone" refers to the locus of primary cell division, the collar of Lillie and Juhn (1932, p. 139).

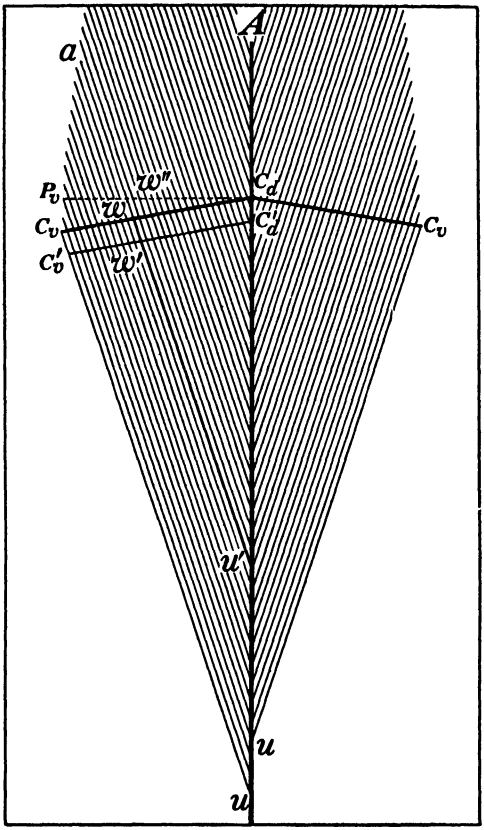



FIG. 1.—Limiting configuration of the locus of simultaneous cell division in the vane of the regenerated feather. The central heavy line represents the shaft; the barbs composing each vane-half are in approximately normal positions but are parallel with each other in each vane-half. Barbs basal to $u$ are removed in each vane-half. The limiting locus of simultaneous growth, the c-isochrone, is the straight line, $C_vC_d$, across each vane-half. The barb-shaft co-ordinates of the c-isochrone are defined as the lengths of barb and shaft from point of c-isochrone incidence to point of union of the barb with the shaft. The limiting c-isochrone co-ordinates are that $C_vu$ shall equal $C_du$, $wu'$ shall equal $C_du'$, and similarly for every barb along the locus $C_vC_d$. A second c-isochrone, $C'_vC'_d$, will be parallel with $C_vC_d$, and is therefore separated from $C_vC_d$ by equal lengths on all barbs and the shaft; thus, $C_vC'_v = ww' = C_dC'_d$. Compare these relations with the germ represented in text Figure 2. $P_vC_d$ is the approximate configuration of a pigmentation boundary imposed simultaneously from ventral to dorsal limits of the germ and also simultaneously with $C_vC_d$.

growth by cell division in the germ. Since this locus of embryonic growth occurs as a straight line, with uniform co-ordinates, a second c-isochrone, $C'_vC'_d$, will be parallel to $C_vC_d$. The second c-isochrone, $C'_vC'_d$, is parallel to the base, $C_vC_d$, of the isosceles triangle, $C_vuC_d$, and must therefore intercept equal lengths on $uC_v$, $uC_d$, and all lines (barbs) parallel to $uC_v$. In terms of axial growth, successive c-isochrones intercept equal increments of growth by all barbs *and the shaft*, and these equal growth increments are laid down in the same time interval. If we accept the usual assumptions respecting growth in the germ, the axial rate of growth of all elements (barb and shaft primordia) in process of growth at any instant must be uniform.[3] This fact must be taken into account by any theory of growth relations in the developing germ.

According to Lillie and Juhn (1932), the shaft primordium of the definitive feather is a bilateral structure, the two halves of which are coextensive with the circumferential base of the germ. The definitive shaft is formed by concrescence of the bilateral primordia. The growth centers of the individual barb are assumed to be transposed from their site of origin to their site of union with the shaft proper by the ventrodorsally directed motion of the shaft primordia. The assumption of bilateral origin of the shaft accounts, therefore, for fixed barb growth centers and the transposition of these individualized centers around the base of the germ.

Considered without reference to other relations, simultaneously uniform axial increments might be accommodated to the theory of development formulated by Lillie and Juhn, although assumptions of a difficult and complex order are required. But the theory of concrescence requires also that a mathematically exact relation shall obtain between the distribution of ridges (barb primordia) in the germ and the distribution of barbs on the shaft of the regenerated feather. Analysis of this relation shows that it does not satisfy the conditions required by theory (see pp. 367–71).

[3] We cannot be certain that growth-rate differentials of an order smaller than we have been able to detect do not characterize the collar of the germ. Growth-rate differentials, of however small an order, would be of definite significance in connection with the reaction differentials described by Lillie and Juhn (1932). We use the term "uniform growth increments" throughout this paper with this reservation in mind.

In the alternative formulation of organization and development required by our results, we assume that the shaft arises from a definitely localized region of the embryonic ring of cells at the base of the germ, and that the axial component of growth by cell division in the shaft is identical with the axial growth component of the barb complement at any instant. These relations are shown in text Figure 2, which represents one-half of a regenerating germ spread into the plane of the paper. The ventral superficial axis of the germ is to the left of the figure; the dorsal superficial axis is to the right. Ridges arise at the face of the ventral triangle, $v-v$, and increase in length and in tangential dimension as they move toward the site of union, $u$, with the shaft. From $u$ the ridges are carried axially from the base of the germ by growth of the shaft.

The axial growth of barbs and the shaft is composed of two phases: growth by cell division and growth by increase in the size of cells thus formed. Lillie and Juhn assume that a constant proportionality obtains between growth by cell division and growth by increase in the axial dimension of cells. We follow them in this assumption, and in text Figure 2 we shall suppose that we are dealing only with growth by cell division.

The region of growth by cell division is bounded in text Figure 2 by the heavy base line, $C_vC_d$, and the lighter line, $h_0$, through barb and shaft bases. We base this formal construction on the observation of Lillie and Juhn (1932), and of Greite (1934) that cell division occurs in part at least within the ridge proper. That portion of the zone of cell division lying between the ventral limit of the germ and the point of barb union with the shaft, $u$, includes only cells entering into barb primordia; that portion lying between $u$ and the dorsal mid-line of the germ gives rise to the primordium of the shaft (only half the shaft is shown in text Fig. 2). In terms of this representation the primordium of the shaft differs from the primordia of the barbs only in that it occupies a fixed position with reference to the zone of cell division and is of greater extent.

The multiplication of cells within the zone of cell division may lead (at least theoretically) to displacement of cells in three dimensions: axially, or at right angles to the base line, $C_vC_d$; tangentially, or in either direction parallel with the base line, $C_vC_d$; and radially,

or at right angles to the plane of the zone as it is represented in text Figure 2.

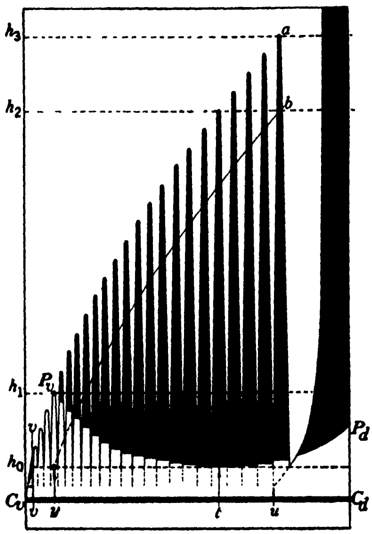



FIG. 2.—Diagrammatic representation of one collar limb of a regenerating feather germ. The germ has been split along the ventral superficial axis (left hand of figure) and also in the center of the shaft (right hand of figure); compare text Figure 8, $V$, ventral, and $D$, dorsal. Barbs and shaft are drawn at right angles to the base of the germ; this representation is strictly diagrammatic and should be compared with text Figure 14 and the germ reproduced as Figure 13, Plate V. The locus of primary growth by cell division is assumed to lie along $C_vC_d$; it extends axially to some level, $h_0$. The level at which barb bases appear is figured as a parallel with the base of growth by cell division; this is strictly diagrammatic. $P_vP_d$ represents a locus of simultaneous pigmentation tangent to $h_0$ at $t$. Ridges join the shaft at $u$. The c-isochrone parallel passing through the ventralmost barb on the p-isochrone, $P_vP_d$, is $h_1$. $P_vy$ indicates the level at which pigmentation occurs in the ventral region of the germ; ridges ventral to this ridge remain unpigmented until they reach $P_v$. The ridge-forming region is to the left of $v$–$v$. We assume that axial growth by cell division proceeds uniformly from $C_vC_d$, or between this base line and $h_0$. A point lying at $y$ follows the locus $yb$ and therefore remains equally distant from the apex $a$ of the completed barb. Ridge bases arising at the ventral surface, $v$–$v$, are transposed toward the shaft by the increasing diameter of every ridge in the collar and by the formation of additional ridges at $v$–$v$. Ridges uniting with the shaft primordium at $u$ are carried from the base of the collar; their spacing on the shaft is not shown.

The configuration of the c-isochrone requires that the axial component of growth be simultaneously uniform through the entire extent of the collar, including the shaft primordium. Any locus of re-

action, $h_0$, parallel to $C_vC_d$, will thus be displaced uniformly from $C_vC_d$ on all barbs and the shaft during a given interval of time. Completion of growth of any individual barb and corresponding shaft level occurs simultaneously at some point, schematically indicated by $u$. A "mark" simultaneously imposed upon any barb and the shaft is therefore at completion of growth equally distant from the point of union, $u$, of that barb and the shaft. The relation of barb and shaft co-ordinates, $b = s$, found in the regenerated feather is thus accounted for by the assumption of simultaneously uniform growth by cell division in the axial sense.

The tangential (ventrodorsal) motion of ridges is referable to two observable evidences of growth: (*a*) ridges are formed continuously at the face of the ventral triangle, $v-v$; and (*b*) with increasing age of ridges the initial tangential dimension is increased. The cumulative growth represented by formation of ridges and the increase in tangential dimension of ridges determines the rate at which ridge bases reach the point of union, $u$, with the shaft primordium. In the fourth part of this paper we attempt to formulate the tangential motion of barbs more definitely in terms of growth vectors; for the present we need only account for the obvious fact that barbs, as individual elements of the germ, describe a ventrodorsal "motion" from site of origin to site of union with the shaft.

In connection with text Figure 1, it was pointed out that the locus of simultaneous pigmentation in the vane of the regenerated feather (represented diagrammatically by $P_vC_d$) is not, as a rule, identical with the locus of the c-isochrone, $C_vC_d$. The usual deflection of the pigmentation line is such that $b$ is greater than $s$, particularly near and at the margin of the line.

The relation of pigmentation lines and c-isochrones in the definitive feather corresponds to differing loci of reaction in the germ. With the locus of growth by cell division, $C_vC_d$, text Figure 2, as base, the locus of simultaneous pigmentation is represented (generally) by a curve, $P_vP_d$. This representation is strictly in accord with the observations of Lillie and Juhn; the differential displacement of the line of simultaneous pigmentation with reference to the assumed locus of growth by cell division is, however, apparently the

result of other differentials than those immediately referable to the axial component of cell division.

Lillie and Juhn (1932) have shown that there is in individual barbs of the feather germ a gradient in reaction to certain physiological agents, such as thyroxin and the female hormone. This gradient is manifest in the extension of pigmentation or barbulation reaction from ridges near the shaft to ridges of the ventral triangle with increasing concentrations of the agents employed. Lillie and Juhn assume these differentials in reaction to depend for their realization and localization upon differing rates of processes arranged gradientwise in the germ. They attempted to obtain a measure of these differentials by estimates of relative rates of axial growth of individual barbs at successive periods of development. One result of the present study is to show that rates of axial growth cannot be used as measure of the observed reaction differentials.

The reaction differentials with which Lillie and Juhn were concerned are based, as they are careful to point out, upon objective evidence which is not dependent upon differentials in the axial rate of barb growth on the one hand or upon any particular theory of development on the other. The relation of c-isochrones and lines of simultaneous pigmentation in regenerated feathers furnishes additional and exact evidence for the conclusion that pigmentation represents a ventrodorsal gradient of reaction with respect to the c-isochrone as base. We are not concerned in this paper with the specific reaction which may determine the gradient, but only with the geometric order of differentials between c- and p-isochrones.

## II. Uniform Axial Growth Increments

The evidence for uniform axial growth increments described in the Introduction comes mainly from the analysis of configurations in the regenerated feather.[4] The configurations with which we are primarily concerned here are the transversely extended lines of fault (Pl. I), the configuration of marginal contours at the apex (Pl. II), and contour configurations in the main vane of the feather (Pl. III).

[4] We wish to express our cordial appreciation to Mr. Arthur Longini for his continued valuable criticism and generous participation in the analyses included in this as well as the other papers of this series.

The contour configurations are relations between spacing of barbs on the shaft and length of barbs.

The conclusions based upon a study of these configurations are strongly supported by analysis of pigmentation configurations. The main importance, however, of pigmentation configurations is in their bearing on organization of the germ; we have accordingly treated them in some detail.

We have attempted to relate the configurations in the regenerated feather as directly as possible to organization and development in the germ. The statement of growth relations given in the Introduction is the basis for interpretation of most of the results with which we are here concerned.

Methods for the preparation of material and for making measurements are described in the first paper of this series (Juhn and Fraps, 1936). All feathers are mounted with barbs at right angles to the straightened shaft, and all graphic representations will be referred to similar co-ordinates.

*Isochrones.*—Configurations which have been imposed simultaneously in all reactive elements of the regenerating germ will be denoted *isochrone* configurations or *isochrones.* This convenient term is due to Hardesty (1933), who applied it to describe in the germ or in the vane of the definitive feather "the locus of points which were formed at the same time." Hardesty used the term with reference to pigmentation lines only. The locus of simultaneous pigmentation in the germ, however, is but one of a number of loci of simultaneous reaction, determination, or "formation." We shall accordingly define the *isochrone,* whether in the germ or in the definitive feather, as *the locus of points of simultaneous and identical reaction or determination in homologous reactive centers of the germ.*

The c-isochrone has been defined in the Introduction as the locus of primary axial growth by cell division. We shall define *the locus of simultaneously imposed pigmentation* as the *p-isochrone. Loci of simultaneous fault reaction, or defect,* may be referred to as *f-isochrones.* We cannot be certain of the exact axial level at which fault reactions occur in the germ; the evidence presented later agrees, however, in locating isochrone fault reaction at approximately one or

another parallel to the locus of growth by cell division. We must, nevertheless, make a clear distinction between the c-isochrone, which is defined as the locus of growth by cell division with respect to the axial dimension of growth, or an exact parallel with this locus, and the *approximation* to this configuration which characterizes the fault isochrone.

*Barb-shaft co-ordinates.*—It will be convenient to speak of the barb and shaft intercepts of any configuration, e.g., faults, and the point of union of any barb with the shaft as "barb-shaft co-ordinates." Transverse shaft levels are taken in apicobasal order as abscissas, and barb lengths in shaft-marginal order as ordinates. The term "barb-shaft co-ordinates" will then be the relation (generally the ratio) between a length or segment of barb and a corresponding length or segment of shaft entering into the definition of any configuration; see text Figures 3, 4, and 5.

*Uniform growth increments.*—We wish to make it very clear that we have used the term "uniform growth increment" (or "simultaneously uniform axial growth-rates") as a limiting condition which may or may not be realized in fact. The material at our disposal is not sufficiently precise to allow us to eliminate the possibility that small differentials in axial growth-rate (by cell division) do not exist around the base of the regenerating feather germ. Theoretically, it seems probable that such differences, of a small but well-defined order, may in fact differentiate one region of the germ from another.[5] The theoretical importance of differences in growth-rate, however small such differences may be, is evident from the investigations of Lillie and Juhn (1932). The main propositions of this paper follow as close approximations in any event.

## A. THE FAULT ISOCHRONES

Fault configurations are loci of defect in the structure of barbules, barbs, or shaft in the vane of the regenerated feather (Pl. I). Faults

[5] We have in mind here growth differentials of the order implicit in D'Arcy Thompson's (1917) treatment of accretionary growth in terms of the logarithmic spiral (see also Huxley, 1932). Curvatures of shaft particularly may prove to be of considerable analytic value in this connection. The ventrodorsal reaction differentials of Lillie and Juhn most probably have a basis in growth differentials, and we are inclined to believe that such growth differentials will prove to fall within limits which are certainly not greater than would be required to account for shaft curvatures in terms of growth differentials.

are of common occurrence in feathers and vary over a very wide range. Riddle (1908) and others cited by Riddle have described the typical fault structures, and we need note here only general characteristics.

The frequency of incidence of fault bars is approximately proportional to the rate of growth of the feathers bearing them (Riddle). They occur with greatest frequency in main tail feathers and in flights. In our material (Brown Leghorn mainly) faults of the type shown in Figure 2 of Plate I are more or less characteristic of the secondary flights. The fault is a narrow transverse band of barbule and barb "incision" or kinking; barbules are seldom missing. Barbs and shaft generally show the defect as a sharp depression, more evident, as a rule, on the outer surface of the feather than on the inner.

Fault bars of a rather different type are commonly characteristic of the tail feathers (Fig. 1, Pl. I). These defects often involve a considerable length of barb. Barbules are completely absent along parts of the theoretical transverse line of fault; barbules along other parts of the same line of fault are apparently normal.

Faults may thus be limited to the single collar limb or they may be localized within a collar limb. These localizations are undoubtedly of significance in terms of rates within the growing germ, but we shall not attempt an analysis of relations of this order.

Fault bars may be experimentally imposed upon the regenerating germ. Riddle induced typical fault structures by reduced feeding, feeding of Sudan III, mechanical "crumpling" with probable damage at the base of the germ, and administration of amyl nitrite. Kuhn (1932) produced faults by feeding deficiency diets and suggested also that the locus of reaction was in the zone of growth. We have induced bars in regenerating breast feathers through the action of adrenalin injected subcutaneously. The induced defect (Fig. 4, Pl. I) is a convex bending or arcing of barbs and shaft dorsally from the plane of the feather vane. The "bar" extends over a considerable length of barb, but barbule defects usually associated with extensive faults do not appear in these bars. The locus of incidence of the reaction falls, within very close limits, along the line of c-isochrone parallels. Figure 4 of Plate I does not show the approximately identical barb-shaft co-ordinates, inasmuch as the feather was

photographed at an angle in order to bring out the definite bending of the barbs.

It should be clearly understood that actual lines of fault may vary greatly in barb-shaft co-ordinates from the exact value of the co-ordinates which define a c-isochrone, or a c-isochrone parallel. Thus in Figure 2 of Plate I, fault 7 obviously does not have the same barb-shaft co-ordinates as do faults 6 and 8. The upper and lower boundaries of faults 4 and 5, Figure 1 of Plate I, are likewise of different configurations in terms of barb-shaft co-ordinates.

In general, faults characterized by narrowness of extent upon barbs (and shaft), identity of defect (i.e., barbule defects solely or barb defect of the same order on each barb), and extending completely from margin to shaft, give also the closest approximation to the uniform co-ordinates of the c-isochrone. These characteristics are most probably the result of relatively severe reactions effective for a brief interval of time, as has been suggested by Kuhn (1932).

### 1. THE CONFIGURATION OF FAULT ISOCHRONES

The two important characteristics of the collar isochrone are symmetry in the two vane-halves and identity of barb-shaft co-ordinates. This definition rests on the determination of characteristics of actual configurations in regenerated feathers, and naturally it is never realized perfectly.

The relations subject to analysis are best seen by reference to Figure 3, Plate I. This feather (a major covert over a secondary, Brown Leghorn) bore a number of sharply defined but extremely attenuated "lines" of fault which are not visible after the feather has been mounted with paraffin. The lines of fault may be located, however, by cutting barbs at intervals along the line of defect before the feather is mounted. After the feather is mounted and photographed, diagonals are drawn from shaft to margin of the vane-halves along the lines of severed barb margins.

The co-ordinates of the lines constructed in this manner are determined by measuring the barb and shaft intercepts of the diagonals in terms of the representation given in text Figure 1. These are treated as ratios of barb and shaft lengths. Let $b$ represent the length of barb segment from its point of union with the shaft to the

point at which the diagonal representing the line of fault crosses it, and $s$ the length of shaft segment from the base of the selected barb to the intersection of the line of fault (as drawn) with the shaft. If $b/s = 1$, the line of fault is, within the limitations of method, identical with the c-isochrone as we have defined it. Ordinarily, $b/s$ will not be exactly equal to unity. The deviations of faults of this type from the c-isochrone are usually small, and they are not consistently in one direction.

The lines drawn into Figure 3 of Plate I are constructed at the theoretically exact angle of the c-isochrone. It is evident at once that in this particular case the actual fault lines, excepting only the sharply broken fault 5 (cf. text Fig. 4), do not vary appreciably from the exactly defined c-isochrone.

A more accurate procedure is to determine by measurement the co-ordinates of lines of fault which are visible after the feather has been mounted. We measure, first, the distance of each barb in basal direction from some point slightly apical to the point of incidence of the fault on the shaft, and secondly, the distance from the point of incidence of the fault on each barb to its point of union with the shaft.

The barb-shaft ratios are calculated for every barb carrying the fault, or the data are treated graphically. The data considered here are best presented in the form of graphs.

Text Figure 3 represents the barb-shaft co-ordinates of an "arc" bar (Pl. I, Fig. 4) in a secondary major covert. The barb co-ordinates are located from the base of the barb to the center of the arc forming the fault; the centers of the bent portions of each barb are remarkably well defined and can be located with a high degree of accuracy.

The points of fault incidence on successive barbs in text Figure 3 are represented by disks on the ordinates. The c-isochrone parallel, *VD*, is constructed through the points of fault incidence; points of incidence of *VD* on the shaft and on any barb are therefore equidistant from the point of union of each barb with the shaft. It is clear that the actual points of fault incidence vary but little from the theoretical c-isochrone parallel. One might possibly show by mathematical treatment that for the data given in text Figure 3

there is a small deflection of the points of incidence of the fault toward the margin of the vane-half, but other determinations, comparable in every respect, show slight deflections in opposite sense.

The exact relation with which we are dealing should be made clear in terms of growth of the germ. Regardless of the level at which fault incidence occurs in the germ, the fault represented in text Figure 3 is well defined on the shaft and falls very closely along the c-isochrone parallel, $VD$. Let $a_2u_2$ represent any barb crossed by the line of fault incidence, and let $w_2$ represent the point of fault incidence on this barb. The experimentally determined relation is that $w_2u_2 = u_2D$. Similarly, $w_1u_1 = u_1D$. With reference to the initial line of fault as base, every barb carrying the fault increases equally in length with the shaft until it has joined the shaft. In terms of text Figure 2, a fault which we may assume to have been imposed at the level $h_0$ (or indifferently at $h_1$) must be removed from the base of the germ by exactly the amount as is the corresponding fault on the shaft before union of barb and shaft occurs.

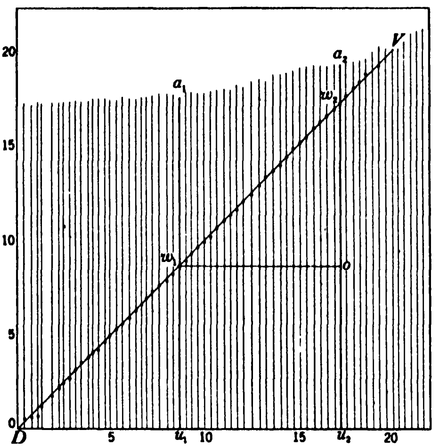



FIG. 3.—Points of incidence of a fault bar on barbs and shaft in the right vane-half of a covert of a secondary, Brown Leghorn capon. Abscissas, distances in basal direction in millimeters from the point of incidence of the fault on the shaft ($D=0$); ordinates, lengths of barbs in millimeters from points of union with the shaft. The diagonal, $VD$, is constructed at the theoretically limiting angle with the line of union of barbs and shaft; for this condition $Du_1 = u_1w_1$, $Du_2 = u_2w_2$, etc.

The fault represented in text Figure 3 is clearly defined on the shaft, as we have observed earlier. If, however, faults were never visible on the shaft, we should be able to demonstrate that equal increments are added to barb and shaft from the base line of growth in the same time interval by analysis of the relation between distances separating barbs and the locus of fault incidence on barbs. In text Figure 3,

$u_1u_2$ is of course equal to $w_1o$, and $w_2u_2 - w_1u_1 = w_2o$, from which it follows that $w_1o = w_2o$. This is to say that the *difference* in lengths of two barbs from the point of fault incidence to the point of union of those barbs with the shaft is equal to the distance separating the same barbs at the shaft. We can understand this relation to mean only that equal increments are added to barb $a_2u_2$ *and the shaft* after barb $a_1u_1$ has joined the shaft at $u_1$.

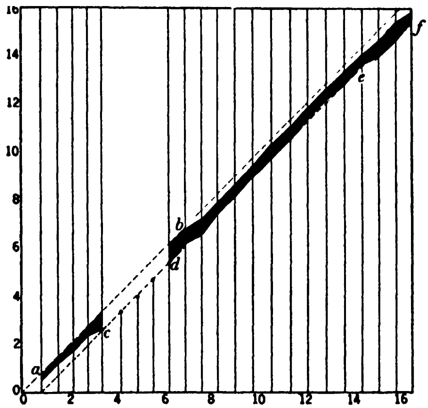



FIG. 4.—Discontinuous fault in the vane of a hackle feather, Brown Leghorn capon. Abscissas, distances on shaft in millimeters; ordinates, distances on barbs from shaft in millimeters. The segment *ab* represents the initial level of fault reaction, segment *cd* an intermediate level, and segment *ef* the basalmost level. The marginal limit of barbs lost between *c* and *d* fall very exactly along the c-isochrone.

The approximate symmetry of fault configurations in opposite vane-halves is evident in such feathers as that shown in Figure 4, Plate I. In many instances faults involve the shaft with sufficient precision to make it clear that there is no appreciable displacement of the point of fault incidence on one side of the shaft as compared with the other. The same conclusion is borne out if we calculate the points of shaft intersection of lines of best fit drawn through points of fault incidence on barbs according to the procedure described in connection with text Figure 3. Lines so constructed for faults in opposite vane-halves are found to intersect the shaft at levels differing by very small amounts on the two sides, and in our material we have been unable to find any consistent difference in transverse level of incidence. The limitations of material and method applying to the definition of co-ordinates in each vane-half separately naturally hold also in location of the level of shaft intersection. We are unable to exclude, therefore, the possibility of a slight but real difference in the level of fault isochrone intersection with the shaft.

Fault bars in many instances are "discontinuous" with respect to

a single c-isochrone locus. Fault 5 of Figure 3, Plate I, is an example of this discontinuity. Text Figure 4 represents a similarly discontinuous fault in one vane-half of a hackle feather, Brown Leghorn capon. Abscissas and ordinates give distances in millimeters from the point of intersection with the shaft of the c-isochrone drawn approximately along the initial region of fault, *ab*.

The several "levels" of fault fall approximately along c-isochrones, as is evident by comparing the boundaries of the heavy black areas, representing total extent of barb defect, with the dotted lines. The most intense region of reaction appears to have been from *c* to *d*. The marginal portions of three barbs in this region are missing; the lengths of the central segments are indicated by the three disks falling along *cd*. A sharply defined incision appears at *c* and another at *d;* all effects between *c* and *d* undoubtedly represent the locus of maximum intensity of the fault reaction.

Fault configurations of this type are clearly evidence for reaction differentials in the collar. The region of fault from *a* to *b* must be due to reaction either at an earlier time or at a higher level on the ridges; similarly, the reaction effecting the fault continued for a longer time along *ef*, or cells closer to the base line of growth were affected through this limited region. The fault represented in text Figure 3 is, in contrast with this situation, practically identical on every barb. This we take to be evidence for relatively intense and simultaneous reaction; it is not evidence that no reaction differentials exist in the collar, a point which we wish to make expressly clear.

### 2. ABSOLUTE AND RELATIVE BARB-SHAFT CO-ORDINATES

The subcutaneous injection of thyroxin alters the normal spacing of barbs on the shaft in many types of feathers (Juhn and Fraps, 1934*b*, Hardesty, 1935). The *absolute* values of the barb-shaft co-ordinates of c-isochrones through modified regions must therefore be altered also (see p. 359). The data presented graphically in text Figure 5 show that the locus of fault through the vane-halves remains unaltered. Points of fault incidence on barbs are represented as ordinates; distances on the shaft from point of incidence of the fault with the shaft, as abscissas. The fault is well defined on the shaft at *D* (=0 on abscissa). The fault does not extend completely

to the margin of the feather but is well defined on barbs 25 mm. in length. The c-isochrone parallel through the points of fault incidence is represented as $VD$.

Barb frequency is defined as the reciprocal of the distance (in mm.) between two barbs and is plotted against shaft lengths in text Figure 5 as the broken curve, $f_s$. It is clear that the relation, $b = s$, characteristic of the c-isochrone, or c-isochrone parallels, is maintained

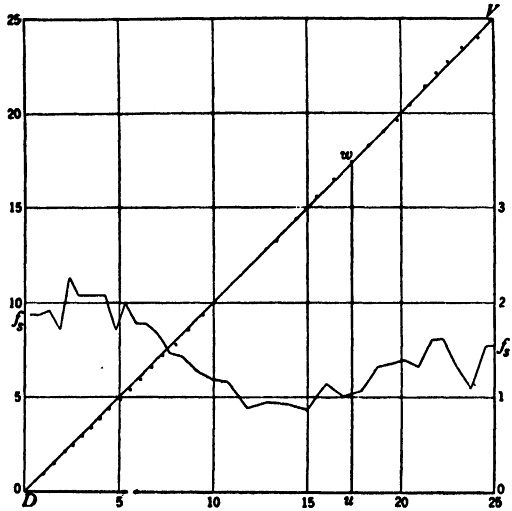



FIG. 5.—Line of fault through the vane of a hackle feather in which spacing of barbs has been experimentally modified (thyroxin). Abscissas, distances on shaft in millimeters from shaft intercept of the line of fault; ordinates, left, distances on barbs from shaft in millimeters; ordinates, right, number of barbs per millimeter on shaft (curve $f_s$). The black disks indicate points of incidence of the fault on each barb; the line of fault is not altered by the spacing of barbs. The relation $Du = uw$ remains constant for all barbs and corresponding shaft segments.

independently of the distribution of barbs on the shaft; $uw = uD$, or approximately so, for every barb bearing the fault.

In a normal hackle feather the spacing of barbs through the region represented in text Figure 5 would be fairly uniform, and $f_s$ would range around 1.5 barbs per millimeter. Since from any point of origin, as $D$, we have a number of barbs more closely spaced than is normal from 0 to 5 mm. (abscissa), it follows that these barbs must have grown by less than their normal length before joining the shaft.

In the region from 10 to 15 mm. on the abscissa the barb frequency is considerably less than normal; and corresponding with this, barbs have grown to a greater length than would otherwise have been the case. It is unnecessary to calculate the exact magnitudes involved here; the important point is that the ratio of barb and shaft co-ordinates, $b/s=1$, is maintained within rather close limits independently of the spacing of barbs on the shaft and independently, therefore, of the *absolute* length of barb and shaft segments.[6]

### 3. THE LOCUS OF FAULT REACTION

The only direct evidence on the locus of fault origin in the germ is due to Riddle, who states that the first indication of fault formation is "a loose union of scattered cells in that part of the *intermediate* cell layer which is forming the barbule cells" (1908, p. 335). The significance of this statement is evident, since it refers the locus of fault reaction directly to the limited region of growth by cell division at the base of the feather germ.

The interference with the growth processes which is commonly recorded as fault bars in the vane of the definitive feather is due, according to evidence presented by Riddle, to reduction of the nutritional level in the regenerating follicle. Riddle associated the reduced level with lowered blood pressure during the night hours of inactivity. Fault bars not infrequently recur with considerable regularity in the feather vanes, the distances separating successive bars corresponding approximately with the expected daily growth of the feather.

It is probable that fault-bar formations generally represent special instances of the principle of thresholds formulated by Lillie and Juhn (1932). Juhn and Gustavson demonstrated (1930) that the quantity of female hormone required to effect a given reaction in different plumage areas is a function of the axial growth-rates of the feathers within those areas. Relatively high concentrations of the

[6] In the material at our disposal (Brown Leghorn capon) we have not a single instance of well-defined faults in feathers which have been subject to relatively high concentrations of thyroxin. It is certainly not improbable that sufficiently high concentrations of thyroxin (or other agents) would bring about alteration in the barb-shaft co-ordinates of faults previously imposed, if only through effects differentially recorded in the second phase of growth.

hormone were required to induce female pigmentation patterns in rapidly growing feathers; lower concentrations induce the same reaction—without causing reaction in rapidly growing feathers—in feathers of lower growth-rates. Reversion to normal pigmentation occurs after shorter duration of the induced reaction in rapidly growing feathers than in slowly growing feathers. The order of reversion with respect to hormone concentration thus varies directly with the axial rate of growth of the regenerating feather.

The frequency of incidence of faults is at least roughly proportional to the axial growth-rate of regenerating feathers, as noted above. Fault bars represent certainly a "reversion" from the normal processes of growth and differentiation of one or another structural element. The order of reversions of this type suggests that fault reactions are referable directly to the principle discovered by Juhn and Gustavson and formulated by Lillie and Juhn, and thus in all probability they involve cell division directly.

Evidence supporting this conclusion comes also, if indirectly, from the experiments of Kuhn (1932) on regeneration of feathers during feeding of deficiency diets. Kuhn fed polished rice which induced defects or "shocks," and brought about recovery by feeding yeast. If the polished-rice diet is continued over a sufficiently long period, there is a definite sequence of interruptions in the normal processes of growth and determination. The formation of the "radioli" is first interrupted, later the formation of barbules with barb defects in the same position, and finally with increasing "shock" no barb cells are differentiated through the affected cross-section. Normal feather regeneration is brought about by feeding yeast.

It is not necessary to suppose that all apparent "faults" represent reversion from a normal level of growth (or determination). It is at least theoretically possible that configurations with typical c-isochrone co-ordinates might be imposed by a sudden acceleration of the processes of growth. The faults induced by adrenalin injection (Fig. 4, Pl. I) are easily imposed upon breast feathers, which ordinarily show very few faults. If the induced reaction accelerated some phase of development sufficiently, we should expect faults to appear first in feathers characterized by slow growth-rates. In either case the origin of altered structures, defective or otherwise, as a result of

altered growth-rate in the embryonic cells of the collar appears to be a special case of the general principle described by Juhn and Gustavson, and as such can be referred most probably to the region of growth by cell division at the base of the germ.[7]

Hardesty (1935) has recently reported the presence of fault bars (barbule defects) which extend across guinea feathers along the usual narrow transverse band. The barbules along such bars are poorly developed, or they may be absent altogether; and in some cases the defect involves the barb also. She notes that the faults are more common and of greater extent following thyroxin treatment, and also that the degree of defect increases with increasing concentration of thyroxin. Hardesty attempted to locate the region of fault in the feather germ with reference to the locus of pigmentation. According to her, "the barbule effect occurs slightly above the line of pigmentation and approximately along an isochrone line" ("isochrone line" refers here to our p-isochrone). She observes that the line of barbule defect is continuous (i.e., symmetrical at the shaft) in asymmetrical feathers and is therefore not symmetrical with respect to corresponding p-isochrones of the regenerated feather.

Hardesty does not present in detail the observations upon which this conclusion is based. If, in fact, the line of barbule defect is above the line of pigmentation, and if, moreover, the defect is due to interference with normal cell division, it seems probable that cell divisions must extend into the region of pigmentation. This conclusion would not be in accord with the statements of Lillie and Juhn (1932) or of Greite (1934), that pigmentation occurs at or near the level at which cell division ceases. The alternative conclusion would be that the fault bar, at least in this particular instance, represents failure of the processes of differentiation rather than of cell growth, or possibly some actual destruction of formed elements, i.e., barbules.

The locus of fault reaction in the germ may theoretically lie through the basal region of embryonic cell division, or reactions determined in this level may become apparent only at higher levels. In certain instances the locus of fault reaction may be specifically

[7] Similar considerations probably hold also for the "fundamental bars" of Whitman (see Riddle, 1908), if these are in fact evidences of a diurnal rhythm in axial growth-rate of the regenerating feather.

the level of barbule formation (Riddle); in others it may lie at considerably higher levels, cutting portions at least of pigmentation isochrones (Hardesty).

In any event, the limiting configuration of faults generally is defined by equal barb and shaft co-ordinates; and regardless of the absolute level of incidence in the germ, the loci of reaction are represented in the vane of regenerated feathers as a system of parallels. The entire system of parallels can most readily be understood by referring them to annular parallels within the zone of simultaneously uniform axial growth by cell division, as $C_vC_d$, $h_0$, $h_1$, text Figure 2. As limiting configurations, the c-isochrone system of parallels is thus evidence for simultaneously uniform axial growth increments by cell division.

B. LIMITING VALUE OF MARGINAL CONTOURS

The marginal contour of the regenerated feather is the continuous line joining the apexes of barbs from apex to base of the feather. In the present discussion we suppose that barbs are mounted at the usual angle of 90° with the shaft, and the c-isochrone has therefore the angular construction of 45° with the line of union of barbs and shaft.

Contour configurations commonly found at the apex of normally regenerated feathers and at subapical levels of feathers regenerated under experimentally modified hormone conditions furnish independent evidence for simultaneously uniform growth increments. In the limiting instances, contour configurations of barb apexes are referable to barb-shaft co-ordinates in the regenerated feather which are identical with the co-ordinates of the c-isochrone as this has been defined previously. It should be emphasized that we are dealing here with limiting configurations.

*Contour tangents* are defined as tangents drawn to the marginal contours of regenerated feathers mounted as described. Such tangents may, of course, be drawn with reference to any point on the curve of marginal contour of a feather, as *ab*, Figure 3 of Plate I, tangent to the marginal contour at the point *p*. We are interested mainly in contour tangents which form an angle approximating 45° with the line of barb-shaft union, the vertex of the angle being directed apically (Pls. II and III).

1. LIMITING VALUE OF CONTOUR TANGENTS AT THE APEX OF THE FEATHER

Apical portions of three feathers are reproduced in Plate II, Figures 5, 6, and 7. Figures 5 and 7 represent equal enlargements of the originals; Figure 6 represents one-half the enlargement of Figures 5 and 7.

*Initial* or *apical* c-isochrones are c-isochrones drawn from the apexes of the primary barbs when these lie in the line of barb-shaft union. These are indicated by "0" in the figures of Plate I; c-isochrones "10" in the same figures are drawn from shaft intercepts 10 mm. from apexes of the primary barbs.

The limiting configuration at the apex is that in which a number of barb apexes fall along the line of initial c-isochrones. These are the apexes between apexes of the primary barbs and points indicated approximately by *i* in Figures 5 and 7 of Plate II. Many feathers are characterized by "acute" apexes in contrast with the right-angle formations shown in Plate II, Figures 5 and 7; typical of these is the hackle feather (Brown Leghorn capon) reproduced as Figure 6, Plate II. Very few barb apexes lie along initial c-isochrones in extremely "acute" formations.

Of more significance in the present connection is the fact that (in our experience) the 45° contour tangent—the initial c-isochrone—represents the limiting configuration, in that barb apexes below the primary barbs may lie along this isochrone but never extend through it. We shall refer to "limiting" apical configurations strictly in this sense.

The significance of the limiting apical configuration is made clear by an important observation due to Lillie and Juhn. These authors pointed out that two distinct phases are to be recognized in formation of the initial complement of ridges in the collar. During the first phase a number of ridges are formed approximately simultaneously; Lillie and Juhn estimate that in the case of the Brown Leghorn breast feather "about 25 barbs form practically simultaneously on each side and that the remainder arise *seriatim* in the ventral field" (1932, p. 139). During the second phase of ridge origin, individual ridges arise serially from the lateral surfaces of the ventral triangle (text Fig. 2.)

Actually, we cannot know that ridges of the first phase are laid

down simultaneously, since theoretically any number of ridges may be formed in a definite time order in the wall of the germ before initiation of axial growth. It is nevertheless probable that a certain number of apicalmost ridges originate simultaneously, in agreement with the observation of Lillie and Juhn that germs in the earliest stages of ridge formation may show either no ridges or a considerable number of ridges. These are the ridges which, as barbs in the regenerated feather, define the limiting contour at their apexes.

The relations involved in the limiting configurations are (growth) relations between barb and shaft lengths and are given in text Figure 6. The apexes of the primary ridges are represented at $A$; the basal level of the vane-half section at $B$. Marginal contours are represented by $Am$ and $Am'$. Barbs $a_1u_1$ and $a_2u_2$ are drawn at right angles to the line of barb-shaft union, $AB$; the initial c-isochrone, or limiting contour tangent, is repiesented by $C_0$.

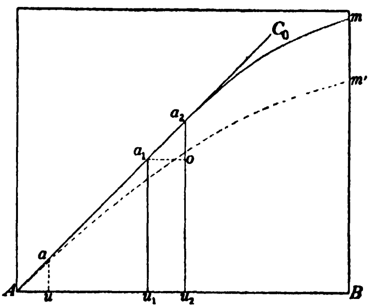



FIG. 6.—The geometric relations involved in the limiting contour formation at the apexes of feathers. $AB$, line of barb-shaft union; apex of the primary barbs at $A$. Barbs are represented by $a_1u_1$ and $a_2u_2$; assumed length of the primary barb, $au$. $C_0$ is the initial c-isochrone (c-isochrone o of plates). Limiting marginal contour, $Am$; "acute" marginal contour, $Am'$. The limiting value of barb-shaft co-ordinates is that $Au_1$ shall equal $a_1u_1$.

In the limiting configuration, the barb apex, $a_1$, falls along the contour tangent, $C_0$. Let $u_1$ represent the point of union with the shaft of the barb $a_1u_1$. It follows that $Au_1 = a_1u_1$. If we assume that barb apex $a_1$ was laid down simultaneously with apexes of the primary barbs at $A$, it follows that the *total* growth (and presumably also rate of growth by cell division), of the barb $a_1u_1$ is exactly equal to the total axial growth of the primary barbs, $Au$, plus the definitive shaft segment $uu_1$.

The limiting barb-shaft relation at the apex of the feather can be generalized as follows: *The length of any barb in the simultaneously formed initial complement is equal to the length of the primary barbs plus the length of shaft segment lying between the base of the primary*

*barbs and the point of union of the given barb with the shaft.* This relation is completely accounted for on the assumption of simultaneous origin of barb apexes falling along limiting contour tangents, and uniform axial growth increments at points of correspondence in barb and shaft. We have pointed out earlier that we cannot be absolutely certain of simultaneous origin of barb apexes, but we come to exactly the same conclusion if we assume the apexes of barbs falling along the initial c-isochrone, $C_0$, to have been laid down before initiation of axial growth.

Lillie and Juhn consider the shaft proper to begin at the bases of the two primary ridges, and we have recognized this distinction in the foregoing relation of barb-shaft co-ordinates. The distinction is clearly true of the shaft as a primary structure. Greite (1934), however, states that the site of the future shaft is definitely localized before its formation as an independent structure, and he notes the reversed symmetry of barbulation on the two primary ridges as evidence for this conclusion. In calculating the barb-shaft co-ordinates of simultaneously formed apical barbs, we consider the two primary barbs to be components of shaft length. This procedure does not necessarily imply that the shaft represents simply fused barb bases (cf. Strong, 1902); it merely locates the point with reference to which the length of "shaft" is identical with the length of simultaneously formed barbs subsequently joining the shaft.

The validity of our interpretation of limiting apical configurations is not, however, dependent upon the relation involving apicalmost barbs only, since we can arrive at the same geometric (and growth) relations in terms of any two barbs the apexes of which lie along the initial c-isochrone. Let $a_1o$, text Figure 6, be the distance between two barbs at their points of union with the shaft, $u_1$ and $u_2$. The difference in lengths of these barbs is $a_2o$. But since the initial c-isochrone, $C_0$, is drawn at 45° to $AB$, it follows that $a_1o = a_2o$; that is, the difference in length of barbs $a_1u_1$ and $a_2u_2$ is equal to the length of shaft, $a_1o$ (or $u_1u_2$), between the points of union of these barbs with the shaft. This relation is directly accounted for if points of correspondence in the two barbs and the shaft, i.e., on all c-isochrone parallels, increase in length by simultaneously uniform increments.

If barb apexes fall along an "acute" marginal contour, *Am'*, text Figure 6, the difference in length of any two barbs is less than the length of shaft between the points of union of these barbs with the shaft. We take this difference to mean that barb apexes composing marginal contours other than limiting contours have been formed in a definite time sequence, only the two primary barb apexes having been laid down simultaneously.

Marginal contours resulting from the serial and individual formation of barb apexes in dorsoventral order are characterized by con-

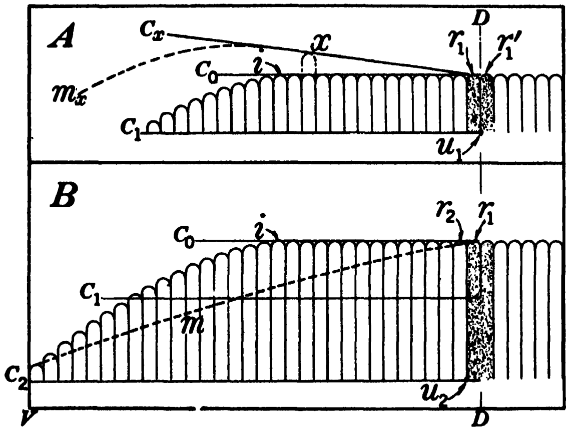



FIG. 7.—Diagrammatic representation of the germinal relations involved in formation of limiting apical configurations; *A* represents a somewhat earlier period than *B*. Ridge apexes formed simultaneously lie along $C_0$; $r_1$ and $r_1'$, primary ridges; *i*, the ventral-most ridge of the initial collar complement. The union of primary ridges to form the shaft proper occurs at $u_1$ along the collar isochrone $C_1$. Similarly, the next barb joins the shaft at $u_2$ along the c-isochrone $C_2$. Compare with text Figure 8.

tour tangents of greatest slope nearest the primary barb apexes. The *rate* of barb formation, then, proceeds most rapidly in the region of the site of the primary ridges, decreasing with completion of the collar complement (text Fig. 9, curve 3, p. 348). The acute apical contour is therefore due to a quantitative variant in the same relations which define the limiting contour tangent.

The germinal relations which correspond to the apical contours of regenerated feathers are represented diagrammatically in text Figures 7 and 8. In text Figure 7*A* we suppose ridges to have formed

simultaneously in opposite directions from a primary pair of ridges, $r_1$ and $r'_1$. These are the ridges lying between $i$ and $r_1$ in the left collar limb, and the apexes of which fall along the initial c-isochrone, $C_0$. If we assume that some small degree of axial growth has been accomplished at this stage of development, we may represent the locus of growth by cell division by c-isochrone $C_1$. Ridges to the left of $i$ are figured as of decreasing length in correspondence with increasing time intervals separating the origin of their apexes.

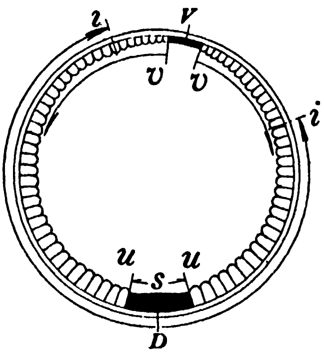



FIG. 8.—Transverse section at the base of the feather germ, diagrammatic. $D$, dorsal limit of the germ; $V$, ventral limit. In formation of the initial complement, barbs are laid down from $D$ to $i$ in each collar limb. In the main vane region barbs are laid down at $v$; tangential growth carries barbs from $v$ to $u$ in each collar limb. The shaft primordium is indicated by $S$. This region is initially defined as the point of union of the primary ridges; it increases in the direction of the arrows at $S$, and at the base of the feather comes to involve the entire circumference of the collar.

Excepting only those ridges formed simultaneously, ridges originate in dorsoventral order until the collar has a complete complement; the extension of ridges around the wall of the germ is therefore from the dorsal axis, $D$-$D$, toward the left-hand limit of the diagram. The *motion* of individual ridge bases is the reverse of the order of ridge formation, i.e., from ventral to dorsal collar positions.

The relations shown in text Figure 7$A$ are emphasized by reference to text Figure 8, which represents a transverse cross-section at the base (approximately the level $h_0$ of text Fig. 2) of a regenerating feather germ after formation of complete collar complements. The ventral superficial axis is at $V$; the dorsal superficial axis, at $D$. At the stage of development assumed for the figure, the ventral triangle (between the ridges, $v$ and $v$) and the shaft primordium, $S$, are definitely localized.

The initial collar-limb complements are laid down in the wall of the germ from $D$ to $i$, either simultaneously or in a definite time order. If the ridges are formed simultaneously, $i$ of text Figures 7 and 8 will correspond with $i$ on the limiting marginal contours of

Plate II. The completion of the collar complement (from $i$ to $v$, text Fig. 8) proceeds at a rapid, but decreasing, rate (cf. text Figs. 9 and 10). After completion of the collar complement, ridges form serially from the lateral surfaces, $v$, of the ventral triangle; and the individual ridge bases "move" from $v$ to $u$, at which point they join the shaft and are carried out of the collar.

Given an initial collar complement of simultaneous formation (text Fig. 7*A*), we must account for the limiting apical configuration in the regenerated feather. We must also recognize that the limiting configuration of apical contours is independent of distances between barbs on the shaft, a point sufficiently emphasized by differences in spacing of barbs on the shafts of the feathers reproduced as Figures 5 and 7, Plate II (these feathers are equally enlarged).

If the apexes of simultaneously formed ridges (those between $i$ and $r_1$) have been laid down along a line parallel with the lines of simultaneous growth, $C_1$ and $C_2$, text Figure 7, and axial growth (by cell division) is simultaneously uniform through all stages of growth, no displacement of the initial c-isochrone, $C_0$, can be effected. This isochrone will continue to rise as a parallel above the zone of growth by cell division.[8] After a definite interval of axial growth, the two primary ridges, $r_1$ and $r_1'$, fuse at their bases, $u_1$, to compose the apex of the shaft proper (Fig. 7*A*). The point of union of the primary ridges is similarly raised above the locus of growth by cell division ($C_2$, Fig. 7*B*) to exactly the same degree that lengths are added to all ridges lying along the base isochrone $C_1$ of Figure 7*A*. When the second barb (in the left collar limb) joins the shaft at $u_2$ (Fig. 7*B*), the length of this barb will be exactly equal to the length of the primary ridges plus the length of definitive shaft lying between $C_1$ and $C_2$. If the simultaneously uniform growth relation is maintained, the apexes of all barbs of the simultaneously originating complement must thus come to lie on the limiting contour tangent.

If the representation shown in text Figure 7 corresponds with actualities in the regenerating germ, it is clearly immaterial at what

[8] It is necessary to realize that we are speaking here of growth increments due to cell division; the second phase of growth is apparently subject to differentials which temporarily "distort" the c-isochrone parallel, but at completion of this phase of growth the original relation of parallels is restored.

level union of the primary ridges, $r_1$ and $r_1'$, occurs, or at what level any of the simultaneously originating complement of ridges shall join the shaft. We have pointed out earlier that the locus of fault reaction (simultaneously imposed on all growing elements) is likewise independent of the spacing of barbs on the shaft or of the *absolute* barb-shaft co-ordinates which define that locus in the regenerated feather.

The simultaneous origin of ridges, whether at the apex of the feather or at other levels, is obviously the limiting "rate" of ridge formation. The initial collar complement need not, however, be formed simultaneously. Assuming that axial growth is under way at the time of formation of the primary ridges, and that ridge apexes form at the same level with respect to the locus of growth by cell division, the serial origin of ridges ventrally from the primary ridges is represented by the contour, $m$, text Figure 7*B*. The level at which $m$ cuts the ridges of the previously described simultaneously formed complement is the locus of the serially formed apexes. The distance between $C_0$ and $m$ along the axes of successive ridges in dorsoventral order represents exactly the length to which the primary ridges have grown at the time of formation of sucessive ridges. The uniform axial rate of growth of formed ridges by cell division and increasing time intervals between origin of successive ridges thus account for the acute marginal contour of the regenerated feather.

One additional point should be touched on briefly in connection with the simultaneous formation of ridges. If the collar is subject to differentials in axial growth-rate (by cell division), the apexes of ridges in regions of high growth-rate (as at $x$, text Fig. 7*A*) should come to lie outside the initial c-isochrone with completion of growth. For the relations represented in text Figure 7*A*, the apical contour tangent corresponding with $m_x$ should make an angle of more than 45° with the line of barb-shaft union. We have not yet found such a situation, although we cannot rule out the possibility that small (but not insignificant) differentials of this order do in fact exist (see p. 328). Also, some care must be exercised in the selection of feathers in order to avoid damaged apexes; it is likely that normal "abrasion," however, would tend to accentuate rather than mask contour tangents characterized by angles greater than the usual 45° c-isochrone.

Apical formations are of particular interest in view of the constancy of the limiting contour tangent in feathers of widely differing types, a point noted in connection with the great difference in spacing of barbs in the feathers reproduced as Figures 5 and 7, Plate II. The order of differences in magnitudes entering directly into apical relations shown by these feathers (and a hackle) are represented graphically in text Figures 9 and 10. The necessary data are obtained by application of c-isochrones at the apex and at 1-mm. intervals through 10 mm. from the apex. Methods and procedures are described in the first paper of this series (Juhn and Fraps, 1936).

The curves of text Figure 9 represent cumulative barb numbers at marginal intercepts (solid lines) and at shaft intercepts (broken lines) of successive c-isochrones. The barb numbers are totals for left and right vane-halves in all instances. Distances in millimeters from apexes of primary barbs are given as abscissas; corresponding barb numbers, as ordinates. Simultaneously determined complements are indicated by *i*.

Marginal barb numbers (solid lines) are a measure of the total number of barb apexes which actually form, after completion of all growth, at a given axial level of growth; shaft barb numbers are the total number of barb bases completed at a given level of axial growth.

Curves 1 (solid and broken) are obtained from the Barred Rock feather reproduced as Figure 5 of Plate II; curves 2 (solid and broken) are from the guinea fowl feather reproduced on the same plate. The third set of curves represent the same data for the apical 10 mm. of a hackle feather (not that shown in Pl. II).

Figures 5 and 7 of Plate II are equally enlarged and are therefore directly comparable. In spite of the much more "blunt" appearance of the guinea-fowl feather, the Barred Rock germ actually formed a much larger simultaneous complement (*i* of text Fig. 9); furthermore, the Barred Rock germ continued to form a larger number of barbs (apexes) per millimeter of axial growth than did the guinea germ. But the number of barbs completed at corresponding levels of axial growth is also much greater in the Barred Rock than in the guinea fowl (cf. curves 1 and 2, text Fig. 9). It is largely in consequence of the relatively great degree of axial growth accomplished

during the interval separating the union of successive barbs with the shaft that the guinea feather shows the combination of widely spaced and relatively long barbs from a simultaneous complement initially smaller than that of the Barred Rock. We apparently have here a set of growth relations (axial and tangential) of first-class importance, and possibly also of interest genetically. For the moment, however, it is sufficient to note only the constancy of the limiting marginal contour in feathers differing so markedly in other respects.

The curves for the hackle feather require no particular description further than to call attention to the form of the curve for marginal barb numbers (3, solid line, text Fig. 9) in contrast with the corresponding curves for limiting configurations.

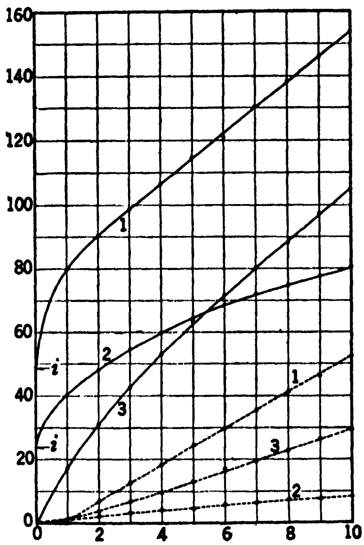



FIG. 9

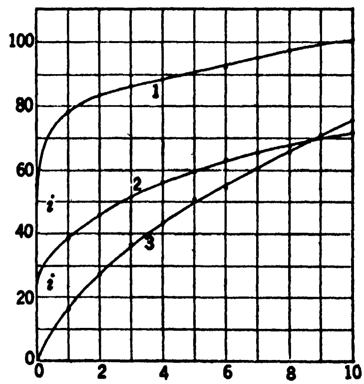



FIG. 10

FIG. 9.—Cumulative barb numbers, both vane-halves; for the marginal intercepts, solid lines; for the shaft intercepts, broken lines. Abscissas, distances in millimeters from apexes of the primary barbs; ordinates, barb numbers. Curves 1 (solid and broken), breast feather, Barred Rock ♀, Figure 5 of Plate 2. Curves 2, breast feather, guinea fowl ♂, Figure 7 of Plate II. Curves 3, hackle feather, Brown Leghorn capon (not that represented on Pl. II).

FIG. 10.—Number of barbs on successive and opposed c-isochrones. Abscissas, distances in millimeters from apexes of the primary barbs; ordinates, barb numbers. The curves are for the same feathers described in connection with text Figure 9.

Curves for numbers of barbs lying on successive and opposed c-isochrones for the three feathers described in connection with text

Figure 9 are given in text Figure 10. The number of barbs thus defined may be taken as the number of barb "loci" simultaneously in process of growth by cell division (Juhn and Fraps, 1936). In these terms the Barred Rock germ (curve 1, text Fig. 10) comes most quickly to approximate "equilibrium"; the guinea germ (curve 2) does so somewhat more slowly. The hackle germ attains its complete barb complement very slowly, but it will be observed that the number of barbs in process of growth (as thus measured) becomes greater than the corresponding number in the guinea germ at a relatively early stage (*ca.* 9 mm. axial level).

### 2. LIMITING VALUE OF CONTOUR TANGENTS AT SUBAPICAL LEVELS

If the relations described for apical configurations are general, we should expect to find that the limiting marginal contour at any level of the shaft is defined also by tangents which are identical in slope with the slope of limiting apical tangents. This expectation could only be realized if barbs might be formed simultaneously after development of the feather is well under way.

That the theoretically possible limiting configuration is in fact realized, or approximately realized, is evident in experimentally induced marginal contours in saddle feathers. The feather reproduced as Figure 8, Plate III, is from the saddle tracts of a Brown Leghorn capon; the apical portion, from the apex to c-isochrone 20 (at 20 mm. from apex), was regenerated under normal physiological conditions. At or near the 20-mm. level the bird was injected with 10 mg. of thyroxin. A normally regenerated saddle feather is reproduced as Figure 9 of Plate IV, and serves as a control.

It is evident, by examination of the close approximation of barb apexes to c-isochrones 24 (left and right vane-halves, Plate III), that the limiting contour tangent previously described in connection with simultaneous apical formations has been very closely approximated in this feather under the action of thyroxin. It is to be observed also that barb apexes in the opposite vane-halves fall upon opposed c-isochrones, although the absolute lengths to which these barbs have grown is very different in opposite vane-halves, and shows, in fact, a reversal in the length relations obtaining before thyroxin action.

The geometric representation of the limiting marginal contour relation at subapical levels is shown in text Figure 11. The marginal contour in its limiting configuration is indicated by *m–m;* a collar isochrone, $C_t$ is drawn through the apexes of barbs $a_1u_1$ and $a_2u_2$. The difference in length of these two barbs approximates as a limit the length of shaft lying between the points of union of these barbs with the shaft. In text Figure 11, $a_1o = a_2o$. This is, of course, the same relation which we have previously described as the limiting relation between two simultaneously formed barbs in the limiting apical configuration.

If the ventral region of the germ can produce but one ridge (in each collar limb) at a time, the contour formation shown in Plate III could not be effected. Lillie and Juhn, however, have observed that the saddle-feather germ is characterized by a considerably greater width of tissue in the ventral region than is, for example, the germ of the breast feather (*v–v*, text Fig. 8). This ventral region bears no apparent ridges. It seems reasonable to believe that it is this undifferentiated ventral field of the germ which, under the influence of thyroxin, differentiates rapidly into definitive barb ridges. If this is true, the parallel between the limiting apical formation and the limiting contour formation induced by thyroxin in saddle germs is complete in both germ and regenerated feather.

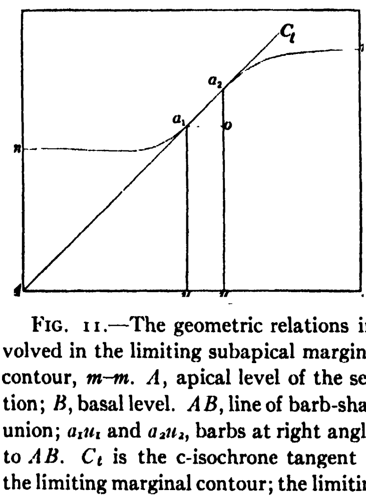



FIG. 11.—The geometric relations involved in the limiting subapical marginal contour, *m–m*. *A*, apical level of the section; *B*, basal level. *AB*, line of barb-shaft union; $a_1u_1$ and $a_2u_2$, barbs at right angles to *AB*. $C_t$ is the c-isochrone tangent to the limiting marginal contour; the limiting relation is that $a_1o$ shall be equal to $a_2o$.

We have found the limiting slope of marginal contours in only a few feathers treated with thyroxin, and all of these are saddle feathers which have been subjected to heavy dosages of thyroxin during regeneration. In most instances the marginal contour does not reach the limiting configuration shown in connection with the feather reproduced as Figure 8 of Plate III. But equally significant

is the fact that we have found no instance in which barb apexes form a marginal contour with a slope greater than that of the c-isochrone.

In order to make evident the relations associated with formation of the limiting marginal contour at subapical levels, we present graphically, text Figure 12, several important relations. The curve *m*, text Figure 12*A*, represents the marginal contour, right vane-half, of the feather shown in Plate III. The contour of an approximate control is indicated by the light dotted line, *m'*. $C_t$ is a c-isochrone drawn through the marginal contour (*m*) at its region of greatest slope, *t;* ordinates, left.

Curve $f_m$ of Figure 12*B* represents the number of ridges formed per millimeter of axial growth from the apex of the feather through the 55-mm. axial level of growth (methods: Juhn and Fraps, 1936). Curve $f_s$ is the number of ridges completed[9] per millimeter of axial growth on corresponding c-isochrones.

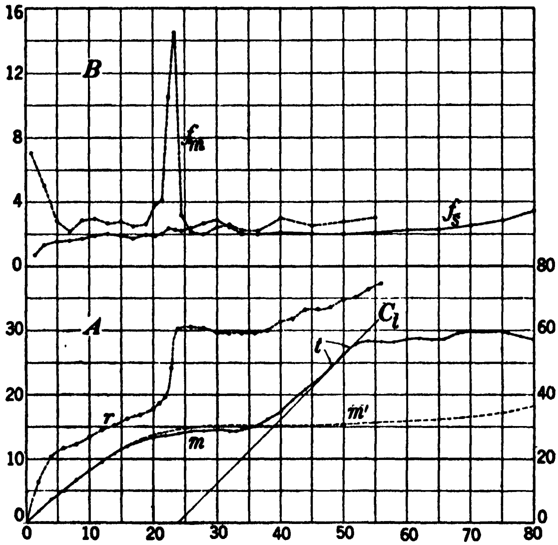
Curve *r* of text Figure 12*A* gives the number of ridges in the collar on successive c-isochrones; ordinates, right hand of figure. The rapidly increasing number of ridges in the collar, beginning approximately with c-isochrone level 20, and reaching a maximum with c-isochrone 24, is referable to the limiting contour tangent, lying also along c-isochrone 24. The actual number of ridges formed per millimeter of axial growth, curve $f_m$ of Figure 12*B*, is very largely cumulative in curve *r* of Figure 12*A*. This is evident in the fact that thyroxin has effected only a small increase in the number of ridges completed per mm. axial growth ($f_s$ of Fig. 12*B*).

The relations shown graphically in Figure 12 represent in all probability an extreme result. Ordinarily, we have found that the number of barbs simultaneously formed by the action of thyroxin is considerably less than in this instance; also, the number of barbs in the collar generally shows a much lesser increase, and tends to fall more rapidly after having reached a maximum value. Finally, the shaft barb frequency ($f_s$, Fig. 12*B*) usually shows, in our experience,

[9] The terms "ridges formed" and "ridges completed" are precise in meaning only with respect to actually defined loci in the regenerated feather as these are determined by c-isochrone constructions. We are not justified in supposing that definitive primordia exist in exact correspondence with these "loci" at the level of growth by cell division in the germ.

a much greater relative increase than does this example (cf. text Fig. 16).

The important conclusion to be drawn from the relations presented in connection with text Figure 12 is that certain contour configurations, experimentally induced, approximate *as a limit* the c-isochrone co-ordinates which we have shown previously to be the limiting value also of tangents at the apex.




FIGS. 12*A* AND *B*.—Action of thyroxin, saddle feather, Brown Leghorn capon, reproduced as Figure 8, Plate III. Right vane-half only. All curves are referable to the single axis of abscissas, millimeters from apex. (Control feather, Fig. 9, Pl. IV.)

Figure 12*A*: *m*, the marginal contour of the modified feather; *m'*, the marginal contour of a control. Ordinates for contours are to the left of the figure and give barb lengths in millimeters. $C_t$ is the collar isochrone passing through the apexes of barbs formed simultaneously in the region *t* of the contour curve, *m*. Curve *r* represents the number of barbs in the collar; ordinates to right of the figure.

Figure 12*B*: the number of barbs formed per millimeter of axial growth ($f_m$) and the number of barbs completed per millimeter of axial growth ($f_s$).

## III. PIGMENTATION ISÓCHRONES

The pigmentation isochrone (or p-isochrone) is the locus of points along which simultaneous pigmentation of the collar complement occurs, or can occur. The locus of simultaneous pigmentation can

be located, at least approximately, with respect to definite structures of the germ, and thus affords a measure of certain general relations which cannot be otherwise defined.

We are interested here mainly in establishing the germinal relations which correspond with the deflection of p-isochrones from c-isochrones in the completely regenerated feather. A second point is to account for the observed differential rate of axial displacement of p-isochrones recorded by Lillie and Juhn (1932).

In general, p-isochrones, or the limiting boundaries of p-isochrones, do not coincide with c-isochrones. We have observed that p-isochrone boundaries may coincide with c-isochrones; this is true, however, only at or near the apex of the feather, and is apparently associated with the formation of simultaneous collar complements. Thus, in Figure 7 of Plate II, one boundary at least of the pigmentation isochrone, that lying along $p_1$, is practically identical in configuration with the c-isochrone. There is, however, a rapid increase in the slope of this boundary with successive periodic pigmentation formations of the marginal series ($p_2$, $p_3$).

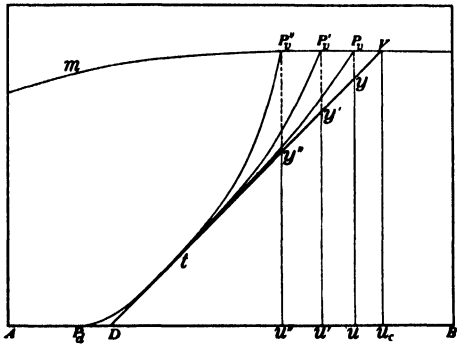



FIG. 13.—Diagrammatic representation of p-isochrone relations found in differing feathers. $AB$, the line of barb-shaft union; $m$, the marginal contour. The $p$-isochrones, $P_vP_d$, $P'_vP_d$, $P''_vP_d$, are drawn tangent to the c-isochrone, $VD$, at $t$. The ventralmost barb in each of these formations is indicated (relatively) by the section $yP_v$, $y'P'_v$, $y''P''_v$. Compare with text Figure 14.

We may assume that the limiting tangent drawn to p-isochrone boundaries is the c-isochrone. In general, however, the p-isochrone is deflected from the c-isochrone in the regenerated feather. The relations with which we have to deal are shown in text Figure 13. $P_vP_d$, $P'_vP_d$, and $P''_vP_d$ represent a series of pigmentation isochrones. These are so constructed in Figure 13 that the c-isochrone, $VD$, is tangent to each p-isochrone at $t$. The grounds for this construction are that the level of pigmentation occurs at or near the apicalmost

limit of cell division in the germ. In any event, $VD$ represents a c-isochrone parallel with reference to which differing p-isochrone curvatures can be compared.

Two characteristics are to be observed in the deflection of p-isochrones. These are:

1. With increasing deflection of the p-isochrone from the arbitrarily constructed c-isochrone there is an increasing length of barb lying between the marginal contour and the point of intersection of the basalmost barb to be pigmented. These are the segments $P_v y$, $P'_v y'$, and $P''_v y''$ of text Figure 13.

2. Corresponding with the increasing deflection of p-isochrones from the tangent c-isochrone, $VD$, there are increasing numbers of barbs between the marginalmost barb on the p-isochrone and the marginalmost barb on the c-isochrone. These are those barbs the apexes of which fall between $V$ and $P_v$, $P'_v$, $P''_v$.

The relations in the germ which correspond with the observed order of deflection of p-isochrones from c-isochrones in the regenerated feather can be made clear by reference to text Figure 14. We are interested mainly in the ventral region of the regenerating germ, since it is here that the deflection of p-isochrones from c-isochrones is at a maximum. The observable relations in a germ in process of simultaneous pigmentation are: (*a*) The ventralmost ridge which is pigmented at its apex, $P_v$, text Figure 14, represents generally the maximum distance from the visible locus of ridge bases, $R_v R_w$. (*b*) There are visible in germs, generally, in process of simultaneous pigmentation, a number of unpigmented barb apexes ventral to $P_v$ of Figure 14. In addition to the visible number of ridge apexes, there are probably a number of "prospective" ridge apexes lying between $v$–$v$ and the actual level of ridge origin.

We have observed also that there is a more or less evident correspondence between the degree of deflection of p-isochrones from c-isochrones in the regenerated feather and the degree of displacement (or slope) of the locus of simultaneous pigmentation of ventral regions from either the base of the germ ($C_v C_w$ of text Fig. 14) or the *visible* locus of ridge bases ($R_v R_w$, text Fig. 14).

The evidence of the preceding section requires that simultaneous axial growth increments (by cell division) be uniform through all

regions of the collar. This conclusion is assumed throughout in the following description of p-isochrone formations.

### A. CONFIGURATION OF P-ISOCHRONES

The induction of simultaneous pigmentation reaction completely across the base of the feather germ is described by Lillie and Juhn (1932). A typical p-isochrone configuration induced by female hormone in a breast feather of the Brown Leghorn is shown in Figure 10, Plate IV. The locus of known simultaneous reaction is the anterior boundary of separation, *p–p*, between the normal black and the induced "female salmon" of the transverse bar. It will be noted that the line of isochrone pigmentation defines rather closely "the line approximately at right angles to the rhachis," which Lillie and Juhn observed to be characteristic of simultaneously induced pattern configurations.

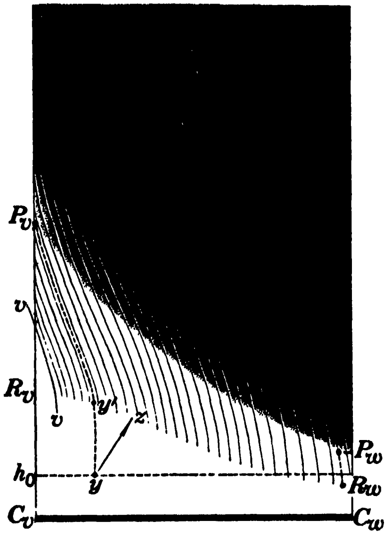



FIG. 14.—Diagrammatic representation of the ventral region of a regenerating feather germ, showing the relations involved in p-isochrone formation. $C_vC_w$, the base line of growth; $h_0$, c-isochrone parallel tangent to p-isochrones (cf. Fig. 13). The locus of simultaneous pigmentation is $P_vP_w$; the locus of apparent ridge bases falls along $R_vR_w$. The ventral triangle region is to the left of *v–v*. The ventralmost ridge to be pigmented is $P_vy'$ (cf. Fig. 13). Ridges to the left of this are without pigmentation in the germ. The assumed vector of growth by cell division is represented by *yz*.

We shall consider here, as examples of simultaneously imposed pigmentation configurations, experimentally induced patterns in the Brown Leghorn and the marginal pattern characteristic of the guinea-fowl secondaries. The experimentally induced p-isochrones (effected by female hormone and thyroxin) are controls from series studied previously by Lillie and Juhn (1932) in their analysis of simultaneous reaction in the germ. The p-isochrone configuration

of the regenerated controls are of particular significance in view of the care exercised by Lillie and Juhn in establishing the conditions for induction of simultaneous reaction completely across the base of the regenerating germ.

### 1. P-ISOCHRONES INDUCED BY FEMALE HORMONE

The feather reproduced as Figure 10 of Plate IV is shown in Figure 11 with barbs mounted at right angles to the shaft. As basis for comparison of barb-shaft co-ordinates in c- and p-isochrones we have drawn paired c-isochrones symmetrically into the vane-halves of the feather; the symmetrically imposed c-isochrones are so located that the c-isochrone is tangent to the most basal of the p-isochrone formations in opposite vane-halves.

The barb-shaft co-ordinates of the p-isochrone in the right vane-half of a feather very similar to that reproduced as Figure 11, Plate IV, are shown in text Figure 15. Points of incidence of pigmentation on barbs from shaft to margin are connected by the line $P_vP_d$. The c-isochrone, *VD*, is drawn tangent to the p-isochrone at *t*.

It is clear that no c-isochrone can be drawn parallel to the p-isochrone, and further, that the p-isochrone is deflected from the c-isochrone increasingly, but not altogether regularly (note particularly the region *ab*, text Fig. 15), from shaft to margin. From shaft to margin in the regenerated feather, corresponds, of course, to dorsoventral in the germ. The portion of the p-isochrone from *c* to $P_v$ (text Fig. 15), particularly, should then be found at a higher level above the theoretical region of embryonic growth by cell division at or near the ventral region in the germ than at intermediate or dorsal levels.

If, in text Figures 2 and 14, $h_0$ represents a parallel to, or within, the zone of growth by cell division, the locus of pigmentation reaction must be approximately of the form $P_vP_d$ in order to give us the general relation found in the regenerated feather (text Fig. 15). The relations here described are thus in agreement with the conclusion of Lillie and Juhn that the locus of pigmentation is at a higher level above the collar in ventral regions of the germ.

There can be little doubt that the zone of embryonic growth by cell division lies basal to the locus of simultaneously induced female

hormone pigmentation (Lillie and Juhn, 1932). In terms of this assumed minimal relation between the c- and p-isochrone, there are a (minimal) number of barb apexes which lie on the c-isochrone but not on the p-isochrone (Lillie and Juhn). In text Fig. 15, these are the apexes lying between $P_v$ and $V$; in the germ, text Fig. 14, the corresponding (visible) apexes lie between $P_v$ and $v$ $v$. On this point, then, the relation between c- and p-isochrones in the regenerated feather are in complete agreement with the observations of Lillie and Juhn.

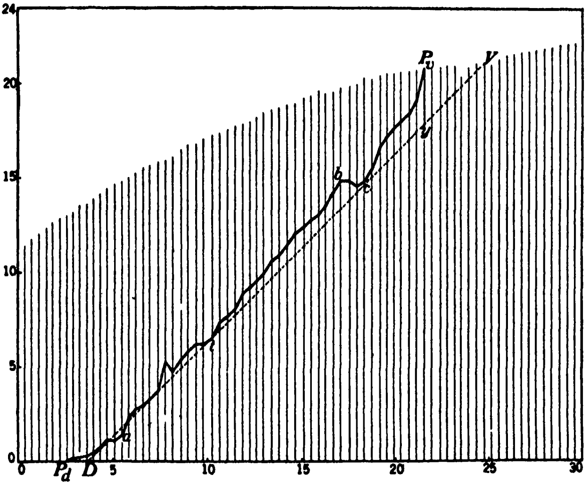



FIG. 15.—Experimentally induced p-isochrone in the right vane-half of a breast feather (female hormone induction). Abscissas, distances in millimeters from anterior limit of the feather section; ordinates, corresponding scale in millimeters. $P_vP_d$ is the locus of simultaneous pigmentation; $VD$ is the c-isochrone drawn tangent to $P_vP_d$ at $t$. $P_vy$, barb segment corresponding to maximum level of pigmentation above the collar. Ridges appearing unpigmented in the germ lie between $P_v$ and $V$. Compare text Figure 14; also Plate IV, Figures 10 and 11.

A second important observation, due also to Lillie and Juhn, is that short lengths of formed ridges at the ventralmost region of breast feathers appear entirely unpigmented. The corresponding barb segments in the regenerated feather are the marginal (barb) lengths lying between $P_v$ and $V$, text Figure 15, and marginal to the c-isochrone, $VD$. No ridge is pigmented in the germ in ventrodorsal order until it reaches a certain minimal length, which in text

Figure 15 is proportional to the barb segment $P_v y$, and which in any event cannot be less for this particular configuration (cf. text Fig. 14 for relations in the germ). Absolute distances in the germ are, of course, much less than in the regenerated feather, since the second phase of growth occurs later in time, and at a higher level, than does the pigmentation reaction.

The general relation between p-isochrones and c-isochrones shown in text Figure 15 are found to hold generally for experimentally induced female hormone patterns in breast feathers of the Brown Leghorn capon. There is, however, considerable variation in the exact co-ordinates of p-isochrones, particularly in degree of deflection shown by p-isochrones in different feathers at marginal limits (ventral in the germ) and in the asymmetry of p-isochrones in opposite vane-halves. We need not go into these relations here.

We have pointed out previously that it is at least theoretically possible that the axial rate of growth of ridges in the ventral region of the germ is more rapid than in dorsal or medial regions, and that we have not yet been able to measure the differentials involved. On these grounds the higher level of pigmentation in the ventral regions of the germ may be referable in part, but only in small part, to a higher rate of axial growth by cell division in these regions.

### 2. P-ISOCHRONES INDUCED BY THYROXIN

In at least some instances, the p-isochrones induced in saddle and hackle feathers by subcutaneous injection of thyroxin approximate more closely in angular configuration the theoretical c-isochrone than do similar isochrones of any type which we have thus far examined. Whether the slight degree of deflection shown by the p-isochrone in the feather reproduced as Figure 8 of Plate III, for example, is due to specificity of the reacting feather follicle or to some peculiarity of thyroxin action is not altogether clear. As is the case also with the female hormone formations discussed above, p-isochrones imposed by thyroxin show considerable variations (cf. text Fig. 16 with Fig. 8 of Pl. III).

The relations between c- and p-isochrones obtaining at the marginal region particularly are identical in order with those described above for p-isochrones induced by female hormone. Differences are

concerned mainly with the less abrupt deflection of the thyroxin p-isochrone near the margin. Corresponding with the smaller deflection of thyroxin p-isochrones from the tangentially drawn c-isochrone (text Fig. 16), there are generally fewer barb apexes lying between $P_v$ and $V$. The marginal segment, $P_v y$, is likewise shorter, as a rule, than the comparable segment in p-isochrones induced by female hormone.

The typical configuration of the p-isochrone is independent of the spacing of barbs on the shaft. In some instances (e.g., Fig. 8, Pl. III) the spacing of barbs is altered but little by the action of thyroxin (text Fig. 12; in this and all similar figures in this paper, $f_s$, the

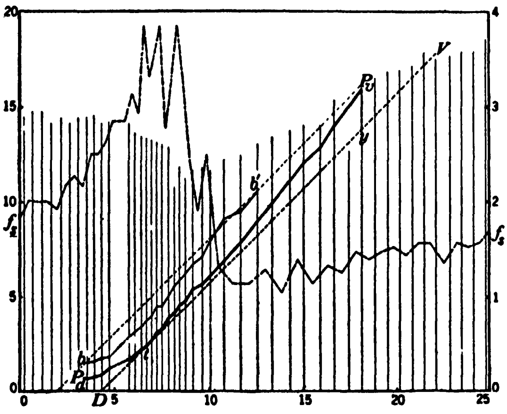



FIG. 16.—Configurations induced by the action of thyroxin in a neck hackle feather (see also Pl. III, Fig. 8); abscissas, distances in millimeters from apical end of the section; ordinates, left, corresponding scale in millimeters. $P_vP_d$, locus of simultaneous pigmentation; $b$–$b'$, locus of barbulation; $VD$, c-isochrone drawn tangent to $P_vP_d$ at $t$. The broken curve, $f_s$–$f_s$, is the curve of barb frequencies, ordinates at right of the figure. Segment of barb corresponding to maximum level of pigmentation at ventral region of the germ is indicated by $P_v y$.

number of barbs per millimeter of shaft length, is the reciprocal of the number of millimeters separating barbs at the shaft.) In text Figure 16 the spacing of barbs (curve $f_s$) is greatly altered, and the changes occur very rapidly. Moreover, the value of $f_s$ is changed in both directions from its normal value in this feather ($f_s$ is around 1.90 in comparable regions of control feathers).

Corresponding with changes in the spacing of barbs are changes

in length of all barbs which were in the collar at the time when thyroxin effected pigmentation. The *absolute* value of the barb-shaft co-ordinates of the different p-isochrones are accordingly different in different instances. If the general form of the p-isochrones remains independent of absolute barb-shaft co-ordinates, we have additional evidence for the conclusion that the *ratio* of barb and shaft co-ordinates of the c-isochrone is constant. If this were not so, the p-isochrones induced by thyroxin would show characteristic shifts at differing regions, and these shifts would be more or less dependent upon changes effected in barb frequencies.

We are, or course, limited here to a *general* relation, since p-isochrones induced by thyroxin are never exactly uniform in different feathers. We are certainly not prepared to say that extreme thyroxin effects may not be associated with modifications, real or apparent, in the constant barb-shaft co-ordinates of the c-isochrone (and thus also of p-isochrones). The subject requires further and exact analysis, and we shall consider it elsewhere.

### 3. THE PATTERN OF GUINEA-FOWL WING SECONDARIES

The most striking evidence which we have been able to find that the angular deflection of the p-isochrones from c-isochrones in the regenerated feather can be referred to differential levels of reaction in the germ is afforded by the rapidly growing wing secondaries of the guinea fowl. The pattern of the guinea has been described by Hardesty (1933), who considered that the theoretical p-isochrone passed through homologous levels of transverse rows of "spots." The transverse rows of spots are not, however, always of simultaneous origin, and this is particularly true in the case of the wing secondaries, which we consider here.

The marginal bar of these feathers frequently takes the form of a relatively extended bar (Fig. 12, Pl. V; and text Fig. 17); and this bar is apparently of simultaneous origin, or approximately simultaneous origin, in the germ. In the left collar limb of the split-germ preparation reproduced as Figure 13 of Plate V, the ventralmost region is in process of forming one of the white bars which will lie marginally in the completely regenerated feather. This ventral region is shown enlarged in Figure 14, Plate V. The portion of vane

represented by Figure 12, Plate V, is the corresponding vane-half of the same secondary from the opposite side of the bird. The marginal bar of the regenerated feather appears in the germ as an unbroken formation, its boundaries are fairly well defined, and it is apparently completely in one or another phase of pigmentation at any given time. We assume, then, that these bars are of simultaneous origin in the germ.

We cannot treat the entire transverse row of formations as of simultaneous origin with the marginal bar, in view of a complete discontinuity in the series at the level of the feather in which the marginal formation shows its greatest deflection from the c-isochrones. This situation is made clear in text Figure 17. We assume that the white spot numbered 14 of the intermediate series (the series lying between axial and marginal series) was formed simultaneously with the marginal bar of the same number (Hardesty, 1933). If an intermediate spot (15, 16, . . . . 22) forms simultaneously with a marginal bar (15, 16, . . . . 22), pigmentation must have occurred at two widely separated marginobasal levels of *the same barb* in the sixteenth period. At the seventeenth period an apparent "duplication" of the intermediate formation has occurred, and we might assume that simultaneous formation of complete transverse series has been re-established. But the same situation arises again by the time the twentieth marginal bar has formed. These relations are of considerable interest, but we cannot go further with them in this place.

The line of simultaneous ventral pigmentation in the germ is approximately indicated by $P_vP_w$, Figure 14, Plate V. The approximate line of *apparent* ridge bases is $R_vR_w$; we cannot exclude the possibility that ridge bases are in process of formation at levels below this line, or that additional ridges are not likewise forming or formed in the ventralmost extent of the collar. The locus of growth by embryonic cell division is assumed to lie at approximately the base of the germ ($C_dC_vC_d$ of Fig. 13, Pl. V).

Corresponding relations in the completely regenerated feather are shown in text Figure 17. The c-isochrone, *VD*, is approximately comparable with the locus, $C_dC_vC_d$, of Figure 13, Plate V, or with some higher parallel, for example, $h_0$ of text Figures 2 and 14. As-

suming that the c-isochrone of text Figure 17 is tangent to the basalmost limit of the white bar, $P_vt$, the relations between the number of barbs in the collar, the number of barbs on this segment of the p-isochrone, and the number of barbs lying between the ventralmost limits of p- and c-isochrones must be at least *minimally* comparable with the visible formations, Figure 14, Plate V.

Ridges which have been formed but which are not cut by the p-isochrone lie between $P_v$ and $y'$ (or $y$). The barb segment, $P_vy$, of text Figure 17 is to be compared with the ridge, $P_vy'$ (or $P_vy$), in

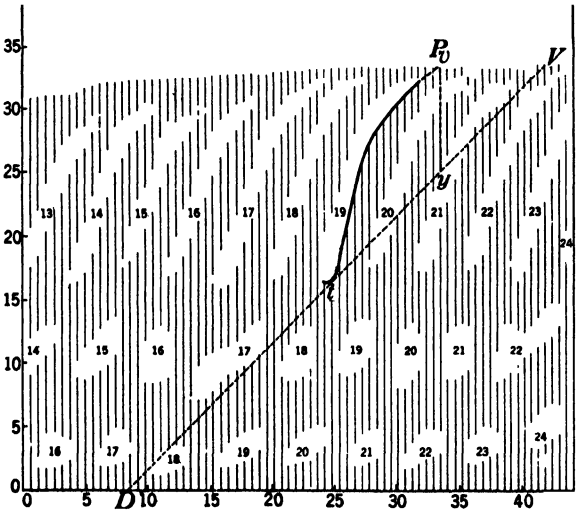



FIG. 17.—Section from a secondary of the guinea fowl wing. Abscissas, distances on shaft in millimeters; ordinates, corresponding millimeter scale. The pigmentation boundary, $P_vt$, is apparently of simultaneous formation in the germ; $VD$, c-isochrone constructed at about the level of $P_w$, Plate V. $P_vy$, relative maximum level of pigmentation in the germ; barb apexes between $P_v$ and $V$ are not pigmented in the germ at time of formation of $P_vt$. See text for description of pigmentation discontinuity between the three numbered series of formations.

the germ. It is clear that the level of pigmentation, $P_vP_w$, Figure 14 of Plate V, lies at a higher level with reference to the visible bases of ridges, $R_vR_w$, at or near the ventralmost limit of the germ than at other regions. The slope of the pigmentation locus with reference to the assumed locus of growth by cell division is, of course, even greater.

The number of barbs between the c-isochrone and the p-isochrone at the margin of the vane (the apexes between $P_v$ and $V$, text Fig. 17) is the least number of ridges which must have been formed or determined in the ventral region of the germ but which do not lie along the simultaneously imposed line of pigmentation reaction ($P_vP_w$, Fig. 14, Pl. V).

### B. THE DIFFERENTIAL GROWTH DISPLACEMENT OF P-ISOCHRONES

The p-isochrones first appear in the regenerating feather germ at differential levels from the visible line of barb bases and are probably even further displaced (differentially, in ventrodorsal order) from the assumed uniform c-isochrone. Growth from the base of the germ displaces the p-isochrone axially, and for a greater or lesser time the *rate* of axial displacement of ventralmost regions is greater than at medial or dorsal regions (Lillie and Juhn, 1932).

If we assume that there is no differential in the rate of cell division in the germ, or that such differentials are too small to account even for the initial differential locus at which pigmentation occurs, it is obvious that the observed more rapid rise of the p-isochrone in ventralmost regions must be referable to some other phase of growth. There are several possibilities which must be considered in this connection, and we shall simply call attention to these without attempting to evaluate quantitatively the observed differential displacements.

1. It is generally agreed that a second phase of growth, during which formed cells increase in size, accounts for the greater proportion of definitive length of the feather elements. It is necessary to assume that *total* growth of the second phase is a constant function of antedecent growth by cell division. The second phase of growth is, of course, initiated only after the conclusion of cell division.

Suppose that $h_1$ in text Figure 2 represents the level at which the second phase of growth is initiated *uniformly* in all elements of the feather germ, and assume also that growth in this phase is uniform at any given level above the base of the germ. The uniform axial displacement of the p-isochrone by cell division will bring ventralmost regions of the formed pigmentation line into this zone before

regions dorsally are brought to the same level. Continued axial displacement of the p-isochrone by growth from the base of the germ will maintain ventralmost regions at higher levels if the *rate of growth* in the second phase is uniform. If rate of growth in the second phase is initiated gradually and increased to a maximum over a period of time (i.e., at higher levels), we should expect to find that ventralmost regions of a pigmentation line would continue to be displaced more rapidly from the base of the germ than are other levels of the same pigmentation line.

As regions of a p-isochrone dorsally removed from the ventralmost region are brought to higher levels within the zone of growth in the second phase, these also should show the accelerated displacement observed at an earlier time at the ventralmost segment of the same or similar p-isochrones. This is, in fact, the case. Finally, as the p-isochrone passes completely through the zone of growth by increasing cell size, we find that the initial displacement of the p-isochrone with reference to the base of the germ is gradually restored.

2. Growth in the second phase is not necessarily uniform in *rate* for all feather elements, even though the total enlargement effected in a cell bears a constant ratio to the original size of the cell. On theoretical grounds it might be expected that barb apexes, which are of smaller cross-section than basal regions of a barb, grow more rapidly during this second phase of growth than do basal levels of the barb. If this in fact occurs, the displacement of ventralmost regions will take place most rapidly from the base of the germ.

3. If we measure in the completely regenerated feather the length of barb segment between two identical p-isochrones, it is found that a greater length of barb lies between these two p-isochrones at their marginal limits, and that the length of this segment gradually decreases as we move toward the shaft. If the p-isochrone represented the primary locus of growth, the barb segments between two p-isochrones would become a measure of differential growth by cell division. If, however, the differential displacement of the p-isochrone represents a purely spatial displacement from the base of the germ at its moment of origin, the length of barb segment between parallel p-isochrones is referable to change in ventrodorsal *position* of the individual barb at successive p-isochrone reactions.

These considerations seem to account adequately for the observed differential rate at which p-isochrones are removed from their site of origin in the germ. There are at least no obvious grounds for believing that the differential axial displacement of p-isochrones following their origin must be referred to growth by cell division.

### C. DIFFERENTIALS IN P-ISOCHRONE REACTION

It was the general conclusion of Lillie and Juhn (1932) that differential pigmentation reactions are the result of rate differentials in the germ; the rate differential in which they were primarily interested was the axial growth-rate of individual barbs. But quite apart from the specific rate differential involved, Lillie and Juhn were careful to show that differentials did exist with respect to threshold of reaction and to the region in which pigmentation was effected. The objective evidence upon which Lillie and Juhn based their conclusions is that with increasing concentration of the reagents employed (female hormone and thyroxin) barbulation and pigmentation extends progressively from dorsalmost to ventralmost regions. Also, with high concentrations of female hormone effective for short times, pigmentation may be restricted to the ventral regions of the germ, and it appears first in the ventral regions in any event.

If the conclusions which we have reached in this paper are sound, differential axial rates of growth by cell division must be of a relatively small order, if they exist at all. That axial growth-rate differentials of an extremely small order may be the decisive factor in differential pigmentation reactions is certainly not disproved by our conclusions. It is not possible, on the other hand, to believe that the relatively great displacement of p-isochrones in the ventral region of the germ is evidence for the rate differentials in question.

We shall assume in this brief discussion of reaction differentials that axial growth by cell division is in fact simultaneously uniform through all elements of the collar. This may appear to involve a contradiction with the results of Lillie and Juhn. This is not the case, however. If it is realized that the lines of simultaneously uniform growth by cell division (the c-isochrones) occur always as a system of parallel lines in the vane-halves, then it must follow that any locus in the completely regenerated feather which is not

parallel with the c-isochrone must involve differentials. The order of differentials involved is measured (if only geometrically) by the deflection between the two lines.

We may put this somewhat differently by saying that any reaction in the germ that is not parallel to the locus of the c-isochrone must involve differentials. If this were not so, all such reactions would compose, in the regenerated feather, a system of parallel lines. It is evident from the examination of simultaneously imposed pigmentation boundaries (text Figs. 15, 16, and 17) not only that the p-isochrones do not compose such a system of parallel lines but also that they show marked differences in different feathers.

Wholly without reference to the specific physiological nature of the differential involved, it is obvious that pigmentation isochrones represent differential reactions in the germ. *If the c-isochrone does in fact represent the locus of simultaneous growth in the feather germ, or is parallel to that locus, any reaction not parallel to the c-isochrone can only be understood to represent differentials (regardless of specific reaction or rate involved) with respect to the c-isochrone itself. Conversely, any isochrone configuration (pigmentation, barbulation) in the vane of a regenerated feather represents the projection of a differential reaction in the germ if that isochrone configuration is not parallel to the c-isochrone.*

We call attention to the fact that pigmentation isochrones are, under certain circumstances, practically parallel with the c-isochrone. The partial configuration, $p_1$, Figure 7 of Plate II, is an instance in point. These formations are undoubtedly associated with the formation of simultaneous collar complements; they are referable to a *uniform* initial displacement from the base line of growth, and also therefore, to uniform reaction differentials.

## IV. The Mechanism of Development

The experimental results (respecting growth particularly) and the conclusions based upon them have a direct bearing upon several aspects of development in the germ. These are, first, the nature of the primordia for shaft, barbs, and the ventral triangle region; second, the motions of growth; and third, developmental relations of a more general nature.

## A. PRIMORDIA OF THE FEATHER

We interpret the evidence from the constant fault configuration and the limiting contour tangents to mean that axial growth in the regenerating feather germ is uniform from ventral to dorsal regions If this is true, we can think of the embryonic base of the feather germ as an annular band of cells in process of growth by cell division. We recognize in this circumferential band (the collar of Lillie and Juhn) three segments which are distinctly differentiated after development of the germ is well under way. These are: (1) the shaft primordium which represents the dorsalmost region of the regenerating germ (text Figs. 7 and 8); (2) a ventral region which is undifferentiated but which, at least in certain instances, can be caused to undergo differentiation into ridges; and (3) the ridges of the bilateral collar complements lying between the ventral triangle and the shaft primordium.

Whether axial growth from the base of the regenerating germ is exactly uniform through the entire region of the collar ($C_vC_d$ of text Fig. 2) or subject to small differentials, it seems to us necessary to recognize the primordia described immediately above as differentiated regions or segments of the collar. This statement of germinal relations is at variance with the formulation given by Lillie and Juhn in 1932. These authors believed the primordium of the shaft to be coextensive with the annular base of the regenerating feather germ. The relations assumed to exist in the concrescence of the shaft primordia of opposite collar limbs are given diagrammatically in text Figure 18.

Text Figure 18 represents a section of a regenerating germ, including the shaft and a few barbs on each side of the shaft spread into the plane of the paper. The dorsally directed flow of the two halves of the shaft primordium, $R$, is indicated by the arrows. The semicircles, $e$, borne by each half of the shaft primordium, represent the growth centers of ridges, which, carried out of the zone of growth after union with the shaft proper, become increasingly separated (black disks), owing to increase in the axial dimension of the cells of the shaft. The points at which growth by cell division ceases are indicated by $u$ for left and right halves of the shaft primordium; these points may

be placed at a higher level without altering the primary relations postulated by the theory of concrescence.

We have pointed out that the c-isochrone relation requires that growth of the shaft be simultaneously identical with that of all barbs in the collar. The obvious difficulty in attempting to relate uniform *axial* increments to the theory of concrescence is that we must assume the *tangentially* directed growth of the shaft primordia to be exactly equal to the axial growth of the barb primordia from their individual growth centers, *e*. The difficulty of this interpretation becomes more apparent when it is realized that extension of the shaft primordium, which comes eventually to occupy practically the entire collar of the germ, would require that an increasingly massive shaft shall be produced by a constantly decreasing length of shaft primordium. This relation is clear by reference to text Figure 8; the shaft primordium, *S*, extends gradually toward the ventral limits, *v*, of the collar, thus gradually reducing the relative length of collar underlying the ridges.

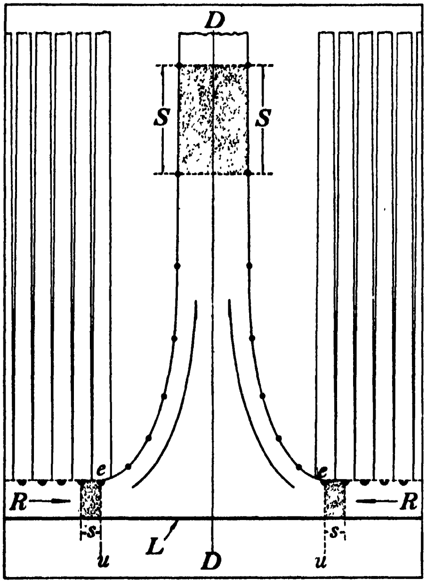



FIG. 18.—Diagrammatic representation of concrescence, based upon text Figure 1 of Lillie and Juhn (1932, p. 129). The central axis of the shaft is in the center of the figure (*D–D*); the dorsally directed primordia of the shaft are indicated by *R*; *L*, lip of the umbilicus. Distance between ridge primordia, *s*; distance between the same ridges after completion of growth, *S*. Growth centers of individual barbs, *e*.

The most decisive evidence which we have been able to obtain in favor of the limited extent of the shaft primordium comes from the relation between spacing of ridges in the collar and the spacing of barbs on the completed shaft. According to Lillie and Juhn, we should expect a constant ratio between the distance separating

dorsalmost ridge centers in the collar and the distance separating barbs on the completed shaft (Lillie and Juhn, 1932).

Let $s$, text Figure 18, be the distance between the growth centers of the two dorsalmost ridges borne by a shaft primordium before concrescence. After completion of differentiation, the distance between these centers, $S$, is the distance between barbs on the shaft. According to the theory of concrescence $S = ks$; this is to say that the distance between barbs on the completed shaft is equal to the distance between the ridges which produce these barbs at their dorsalmost position multiplied by a constant, $k$, which represents the increasing size of cells in the second phase of growth.

It is a safe assumption, borne out by examination of germs, that the distance between ridge centers ($s$ of text Fig. 18) increases with apicobasal formation of barbs through at least the period of growth occupied by the initial ridge complement. Following this period, the distances separating ridge bases in the collar may remain fairly uniform, but there is no evidence of decreasing distances between ridge centers in formation of the main vane.

In terms of concrescence, then, we should expect to find that the distance between these barbs on the definitive shaft increased in proportion to the increasing distance between ridge centers in the collar. We find, however, all manner of curves for the spacing of barbs on the shaft. Several of these are given in text Figure 19. Curve 1 gives the barb frequency (i.e., the reciprocal of distance between barbs) for a Brown Leghorn breast feather; curve 2 represents the barb frequency in a guinea breast feather; curve 3 shows the barb frequency in a hackle feather from a Brown Leghorn capon.

The order of increasing barb frequencies, i.e., decreasing distances between barbs, shown by curves 1 and 2, is clearly the opposite of what we should expect to find in terms of concrescence. If we assume that the ridges which join the shaft primordium from the apex to the fortieth millimeter of shaft growth are uniformly spaced in the collars which produced feathers represented by curves 1 and 2, it is necessary also to assume that these identical lengths of underlying shaft primordia are capable of producing lengths of definitive shaft varying by the values of the ordinates applying to these curves.

The spacing of barbs on the shaft is completely accounted for if

we assume that the individual ridge may join the shaft at any time; it is only necessary to assume that axial growth in this given ridge and in the shaft primordium are uniform as long as the ridge remains in the collar. In our opinion, this identity of growth of ridge and shaft primordium can only be accounted for by the assumption that the shaft primordium represents a limited segment of the collar and is subject to the same growth-rates which at any moment obtain through the remainder of the collar.

If we conclude that the shaft primordium is not a bilateral, tangentially directed primordium in each collar limb, it follows also

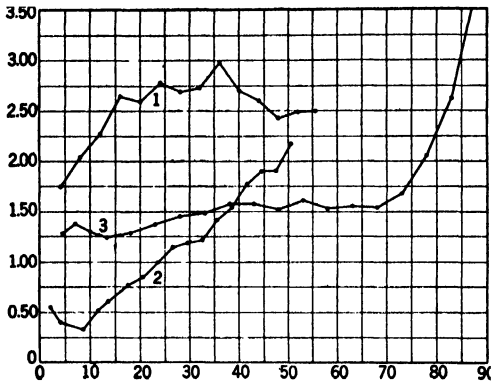



FIG. 19.—Barb frequencies at the shaft ($f_s$) in different feathers. Abscissas, distances in millimeters from apex of feathers; ordinates, number of barbs per millimeter of shaft length (or growth). Curve 1, left vane-half of breast feather, Brown Leghorn capon. Curve 2, breast feather of guinea fowl ♂ (Fig. 7 of Pl. II), averaged from both vane-halves. Curve 3, hackle feather, Brown Leghorn capon, $f_s$ averaged from both vane-halves.

that the individual ridge growth centers postulated by the theory of concrescence cannot be passively carried from their site of origin at the ventral triangle to their point of union with the shaft proper. The alternative hypothesis of localized primordia for shaft and barbs and the growth relations by which we account for the ventro-dorsal translation of the barb primordia make it unlikely that the ridges have at their bases a fixed growth center. From this point of view it is probable that the growth and differentiation properties of the individual ridge are functions of their positions in the collar and

that no fixed growth center need be postulated to account for the properties of the individual ridge.

### B. THE MOTIONS OF GROWTH

The statement of growth given in the introduction of this paper is purely descriptive. The relations involved may now be stated in somewhat more concrete form.

1. The results presented in this paper are evidence for the assumption of simultaneously uniform *axial* growth increments. This is simply to say that the entire base of the collar, including the shaft primordium, grows axially by uniform increments. We assume that the locus of axial growth lies at the base of the germ, $C_vC_d$ of text Figure 2 and $C_vC_w$ of text Figure 14. The grounds upon which we base the assumption of simultaneously uniform axial increments have been sufficiently emphasized and need not be described further here.

2. It is also necessary to assume the existence, in the regenerating feather germ, of a tangential component of growth. This tangential component accounts for the (apparent) motion of ridge bases from their site of origin to their site of union with the shaft. We may assume that the tangential component of growth is imposed at regions apical to the axially growing cells at the base of the germ, or that all cells in process of cell division are characterized by a vectorial displacement ($yz$, text Fig. 14).

If we assume that the axial component of growth is entirely independent of the tangential displacement, tangential growth must then occur at right angles to, and above the annular plane of axial growth at the base of the germ. Referring to text Figure 2, we may assume that the axial component of growth ceases at or about the level $h_0$. If the tangential component of growth is impressed at about this level, the definitive ridge structures will increase in size and be displaced from ventral to dorsal limits of the germ.

At the other extreme, we may assume that all growth by cell division in the feather germ represents a true vectorial displacement. This would be merely to assume that increasing numbers of cells are displaced, not strictly axially from the base of growth in the germ, but at an angle which would represent the resultant of increases in axial and tangential directions.

In any event, it is the tangentially directed vector of growth which accounts for the transposition of ridges from their site of origin at the surfaces of the ventral triangle to their site of union with the shaft. If the tangential vector is relatively increased, ridges will join the shaft more rapidly; if it is decreased relative to axial growth, ridges will join the shaft at a slower rate. The significant point is that tangentially directed growth of cells at the base of the germ, or within the definitive ridge structures, is adequate explanation for the ventrodorsal transposition of ridges.

One other point remains to be made clear: According to the conception of growth which we have set forth here, every transverse level of the completed barb arises at a definite ventrodorsal position in the collar. This can be made clear by reference to text Figure 2. Let us suppose that cells at $y$ represent the apical limit of the region of growth by cell division in the ridge $P_v y$. It is evident that the continuation of axial growth from the base of the germ must displace this level above $h_0$. At the same time, or independently, the tangential motion of growth displaces the point $y$ ventrodorsally. The locus of motion of the level $y$ is then along the line $yh$, text Figure 2. Similar relations may of course apply to the region of cell division below the visible bases of ridges. Thus, in text Figure 14 the level $y$ is displaced in the direction of the arrow to $z$. The apparent motion of barb bases becomes, in terms of the conception of growth given here, a function of vectorial displacements. Every level in the length of a barb is therefore referable to a definite position along the collar in ventrodorsal order. Stated conversely, the formation of successive transverse levels of the individual barb occurs at successive ventrodorsal positions in the collar.

### C. THE ORDER OF DEVELOPMENT

The developmental sequence of barb and shaft formation and the relations between them may now be summarized briefly as follows:

1. The first stage of differentiation of definitive feather elements is the formation of ridges in the wall of the collar. The order of ridge formation is dorsoventral with respect to the future site of shaft formation. The limiting "rate" of initial ridge formation is the simultaneous formation of a ridge complement. The marginal barb

frequency, i.e., the number of ridges laid down per millimeter of axial growth, is very high at the apex of the feather under these conditions. The two primary ridges appear to arise simultaneously in any event, and acute apical formations are referable to serial formation of ridges after formation of the two primary ridges.

2. Axial growth of the feather germ is initiated before formation of the shaft proper; this is evident in the fact that the two primary ridges grow through a greater or lesser length before they fuse at their bases to form the apex of the shaft (cf. Lillie and Juhn, 1932). The future site of shaft formation is determined at the time of origin of the two primary ridges (Greite, 1934). After the two primary ridges fuse at their bases, the single structure thus formed grows for a greater or lesser extent before ridges from the opposite collar limbs fuse serially with it. The spacing of barbs below the point of fusion of the two primary barbs is highly variable in different types of feathers, and is referable to growth relations involving axial and tangential rates.

3. With continuing development of the regenerating feather, the shaft primordium occupies an increasing tangential segment or arc of the collar. Toward the base of the feather, the segment occupied by the shaft primordium increases rapidly in extent, and during formation of the quill the entire annular base of the germ is projected axially as a cylindrical structure.

4. Following the formation of apicalmost ridges of the feather, ridges are laid down serially from a mass of undifferentiated tissue which becomes the ventral triangle of the germ. The ventral region is of variable extent in different feathers; that it may be of considerable extent is evident in the order of ridge formation effected by thyroxin.

5. Uniform axial growth increments obtain from formation of the apex of the primary ridges to the completion of regeneration in all feathers.

6. After the collar of the germ is definitely organized, it is subject to reaction differentials of the order described by Lillie and Juhn. These ventrodorsal differentials are evident in the deflection of lines of simultaneous pigmentation from the uniform locus of axial growth by cell division.

## V. Summary

1. Evidence is presented for the conclusion that axial growth increments in the regenerating feather germ are uniform, or approximately uniform, for all elements in process of growth during any time interval. This conclusion is based upon an analysis of fault bars, normally occuring formations at the apex of feathers, and experimentally induced formations at subapical levels.

2. The relation of simultaneously imposed pigmentation configurations to the base line of simultaneously uniform growth by cell division is described, and the geometric relations between the two loci of simultaneous reaction are formulated as a spatial differential, independent of axial growth-rate differentials referable to cell division.

3. Interpretation of configurations in the feather has required a general statement of the mechanism of regeneration. The primordia of the feather and the motions of growth during development are formulated in terms which account for the properties of the configurations described in this paper.

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# EXPLANATION OF PLATES

All figures are photographs and microphotographs.

## PLATE I

### Fault-Bar Configurations

Typical fault bars in several types of feathers.

Fig. 1.—Main tail feather, Brown Leghorn capon. The faults are characterized by marked reduction, or total absence, of barbules. The fusion of barbs on fault 4, right vane-half, is due to failure of ridges to separate during differentiation.

Fig. 2.—Wing primary, Brown Leghorn capon. The faults occur as defects in barbules and are very limited in axial extent; in some instances slight incisions occur in the shaft. Faults are limited wholly to the right vane-half; primaries generally show marked asymmetry in frequency of faulting in opposite vane-halves.

Fig. 3.—Major covert of secondary, Brown Leghorn capon. Marginal portions of barbs have been removed by cutting along lines of fault before mounting the feather; barbs are then mounted at right angles with the shaft. The several diagonals are drawn to make an angle of 45° with the line of union of barbs and shaft. These diagonals therefore define points equidistant on shaft and all barbs from the point of union of barbs with the shaft. Fault 5 is discontinuous in the mid-region of the vane-half.

Fig. 4.—Experimentally induced fault (adrenalin), breast feather, Brown Leghorn capon. The fault occurs as a well-defined bending of barbs from the plane of the feather vane. The photograph does not show true barb-shaft coordinates, inasmuch as the feather has been inclined to show the bending of the barbs.

## PLATE II

### Apical Contour Configurations

Figures 5 and 7 are two times the enlargement of the feather shown in Figure 6. Apicalmost c-isochrones, o, are drawn in each instance from apexes of the primary barbs at angles of 45° with central axis of the shaft. C-isochrones 10 are drawn from shaft intercepts 10 mm. from apexes of primary barbs. The ventralmost barb in the complement of simultaneously formed barbs is indicated by *i* in each vane-half.

Fig. 5.—Breast feather from Barred Rock ♀; from or near the axis of symmetry of the tract. Unmounted barbs at the apex: left, 12; right, 11. Barbs formed simultaneously: 27 in the left vane-half, 22 in the right vane-half.

Fig. 6.—Hackle feather, Brown Leghorn capon, showing "acute" contour formation at its apex. This feather has been treated with thyroxin, repeated injections in low dosages. The form of the marginal contour is, however, typical of hackle feathers, and the pigmentation induced by thyroxin renders it especially suitable for reproduction.

Fig. 7.—Breast feather from guinea fowl ♂; position in the tract, on or near the axis of symmetry. Unmounted barbs at the apex, 4 on each side. Barbs formed simultaneously: left, 13; right, 11. The increasing displacement of pigmentation isochrones in apicobasal order of their formation is shown by slopes of the lines, $p_1$, $p_2$, $p_3$, drawn tangent to marginal "spots" of the pattern.

[See page 377 for legends for Plates III–V]

PLATE I

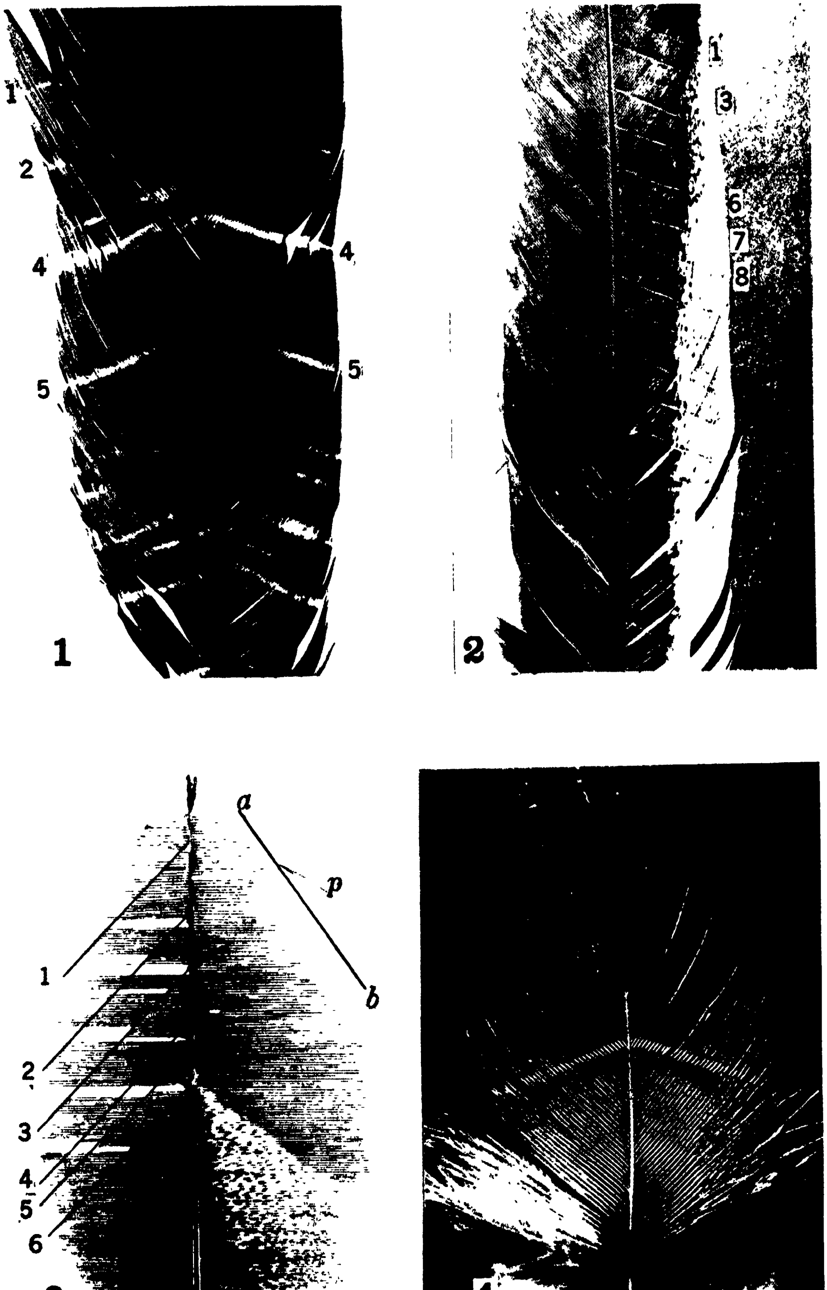
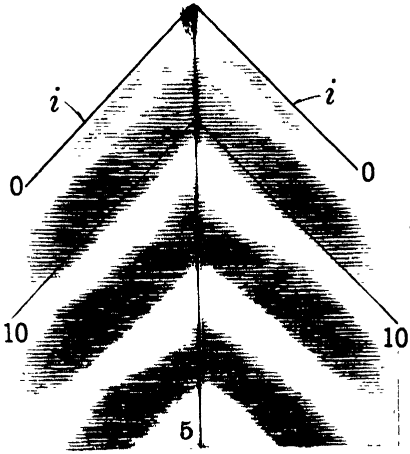
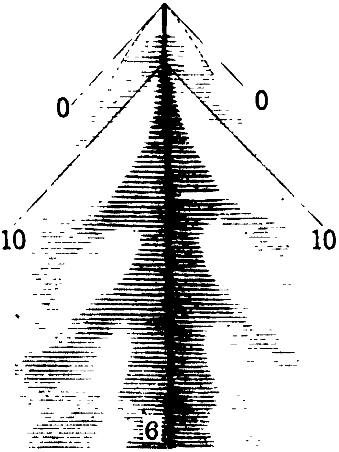


PLATE II



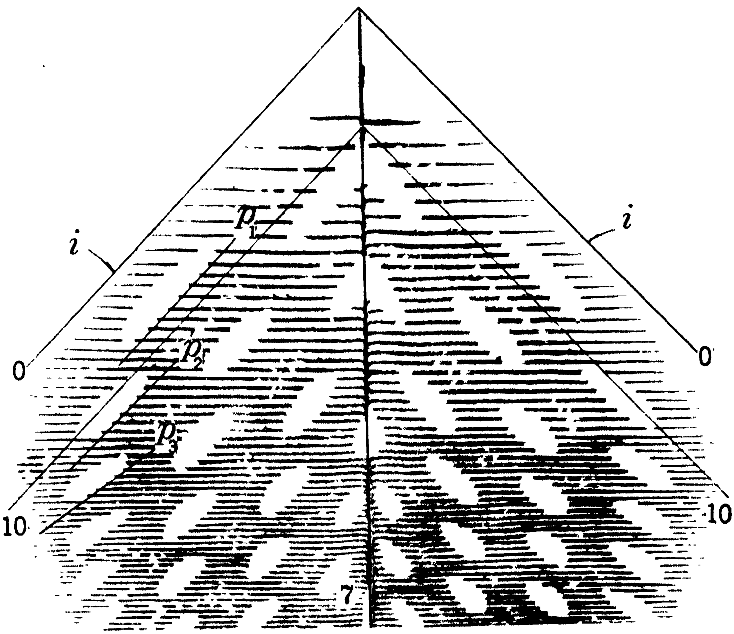



PLATE III

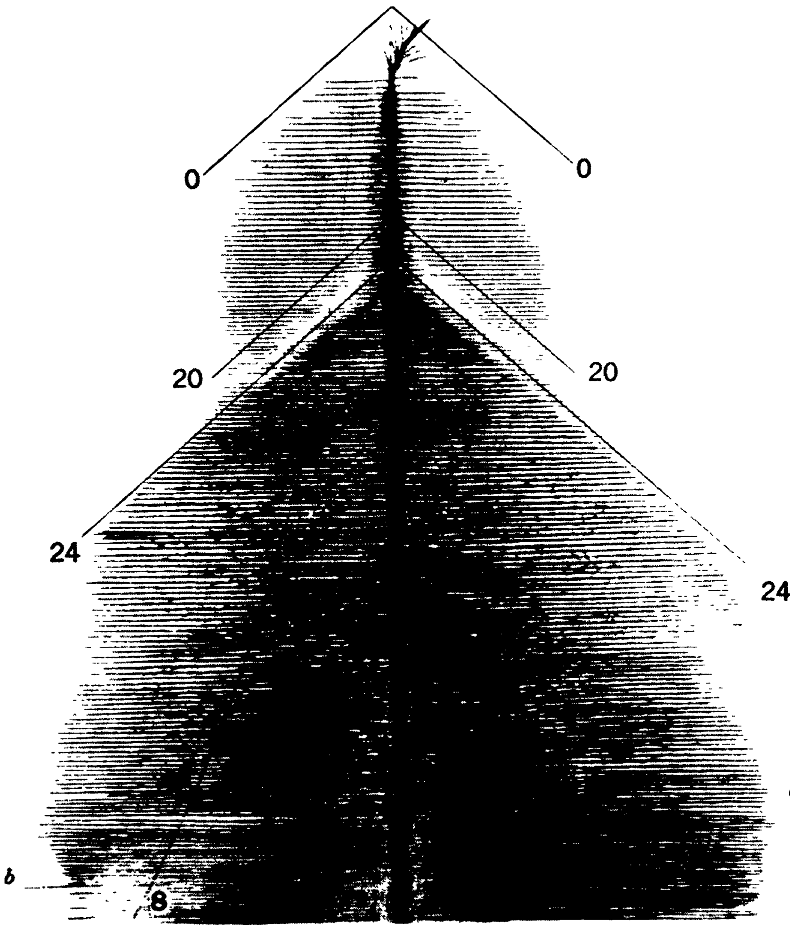


PLATE IV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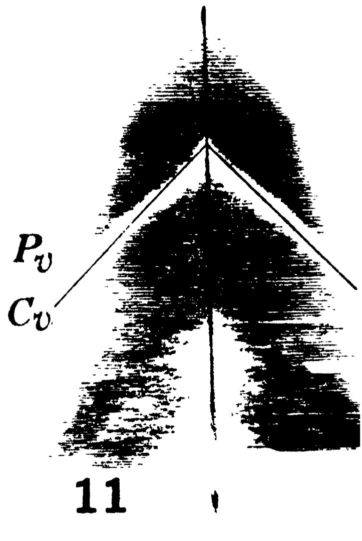


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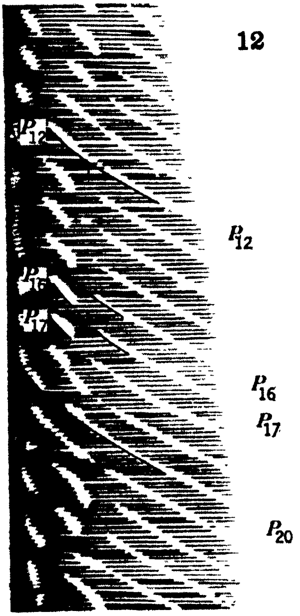
PLATE V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$P_{12}$
$P_{16}$
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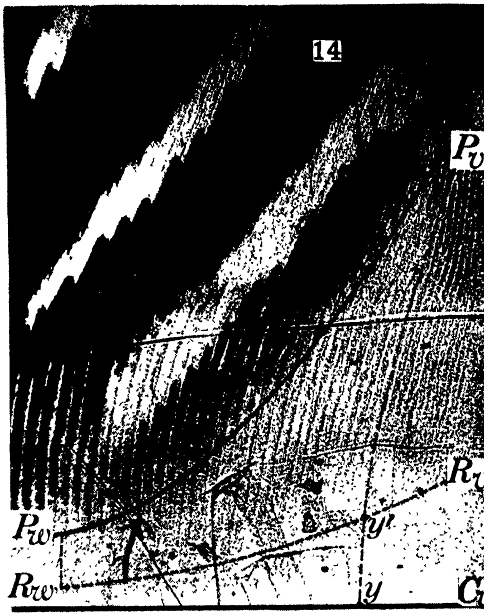
14
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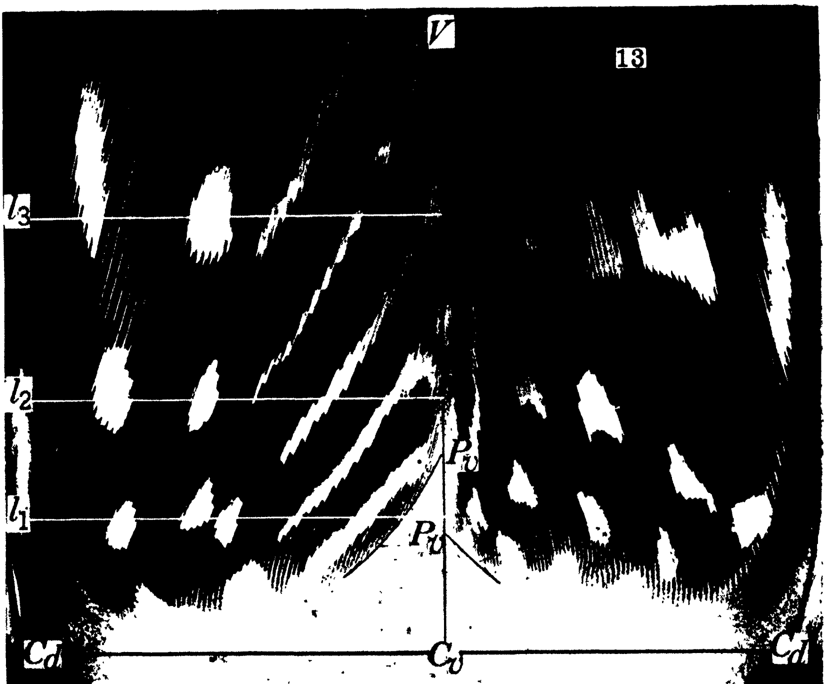
V
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$P_v$
$P_v$
$C_d$
$C_v$
$C_d$

## PLATE III

### Limiting Marginal Contour in Mid-vane Region

Fig. 8.—Saddle feather, Brown Leghorn capon, single injection of 10 mg. thyroxin. Exact position unknown. (Approximate control shown as Fig. 9 of Pl. IV). The first evidence of thyroxin action occurs at c-isochrone 20 i.e., 20 mm. from the apex of the feather. The extension of the ventral triangle or the differentiation of this region has been completed at 24 mm. Barbs formed simultaneously, or approximately simultaneously, are clearly defined as those barbs, the apexes of which lie along c-isochrone 24 (cf. text Fig. 12). Occasional barbs in the "fluff" region extend considerably beyond the normal contours; two of these are indicated at *b*.

## PLATE IV

### Pigmentation Isochrones: Figs. 10 and 11

Fig. 9.—Saddle feather, Brown Leghorn capon; approximate control for the saddle feather shown on Plate III.

Fig. 10.—Breast feather, Brown Leghorn capon, with experimentally induced female hormone pattern. The boundary of simultaneous pigmentation is the apicalmost boundary of the transverse bar, *p-p*.

Fig. 11.—The same feather shown in Figure 10, mounted with barbs at right angles to the shaft. The apicalmost boundary of the bar induced by female hormone deflects from the c-isochrone, $C_v$, in an apical direction; this is the characteristic deflection for simultaneously induced pigmentation lines in feathers of this type. Compare with the pigmentation line induced by thyroxin, Plate III.

## PLATE V

### Relations at the Ventral Triangle, Guinea Fowl Secondary Flights

The three figures represent germs and portion of a secondary flight of the same position in the primary-secondary sequence.

Fig. 12.—Portion from the main vane showing the discontinuity of marginal and medial pigmentation formations. $P_{12}$ is continuous through the median formation. Discontinuity of the usual transverse formation occurs at $P_{16}$ and $P_{17}$ and is completed at $P_{20}$. We limit comparison of germs and regenerated feathers to the marginal pattern, corresponding with the ventral region of the germ.

Fig. 13.—Split preparation of the regenerating germ; the germ has been split along the center of the shaft, which therefore occupies the left and right limits of the photograph. $C_v$–$V$, the ventral superficial axis. $C_dC_vC_d$, the locus of growth by cell division. $P_v$ (left) and $P_v$ (right), ventralmost limits of pigmentation. The horizontals, $l_1$, $l_2$, and $l_3$, passed through centers of medial formations, show the increasing rate of growth of the second phase with increasing level above the base of the germ.

Fig. 14.—The ventral region from the left half of Figure 13, enlarged to show barb bases and the differential level of pigmentation with respect to barb bases. $P_vP_w$, locus of simultaneous pigmentation (corresponding to marginal bars of Fig. 12). $R_vR_w$, approximate locus of visible ridge bases. The base of the section is approximately along $C_dC_v$ of Figure 13. The ventralmost pigmented ridge is $P_vy'$. Apexes of unpigmented ridges fall between $P_v$ and $R_v$; their bases between $R_v$ and $y'$.

# DEVELOPMENTAL ANALYSIS IN PLUMAGE. III. FIELD FUNCTIONS IN THE BREAST TRACTS

(One plate and eight figures)

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THE degree of correspondence between vane-halves of the individual feather varies over a remarkable range. Certain feathers of all tracts are approximately symmetrical with respect to obvious characteristics, such as pigmentation patterns, the length of barbs, the distribution of barbs on the shaft, the distribution of barbules, etc. Other feathers from the same tract show asymmetry in some or all of these elements of structure or configurations of pattern. In some instances, e.g., the primaries, the asymmetry in the vane-halves becomes extreme and is evident in marked differences of pigmentation configurations, spacing of barbs, and mass of individual barbs. In other feathers, e.g., saddle feathers, the asymmetry is of much lesser apparent degree but is nevertheless easily recognizable in one or another respect in most feathers of the tract. These general relations have, of course, long been known and in some instances have received considerable attention from the morphological point of view (e.g., Chandler, 1916).

The distribution of differing symmetry types within plumage tracts has been treated quantitatively by Landauer (1930). Working with the spangled (apical) pattern of the Silver Spangled Hamburg, Landauer established general relations of reversed asymmetry on the two sides of the body. In at least one instance (the wing coverts of the male) he recorded a definite sequence of asymmetry types.

Lillie and Juhn (1932) considered the asymmetry of the individual feather in terms of physiological principles, i.e., in terms of

[1] This investigation was supported in part by funds from a grant by the Rockefeller Foundation to the University of Chicago in aid of biological research; one of the authors (R. M. F.) was the recipient of a grant-in-aid from the National Research Council.

growth and development within the feather germ. Their analysis was based upon the discovery of Juhn and Gustavson (1930) that the threshold of reaction of the individual feather of the male and capon to female hormone is proportional to the axial growth-rate of the feather. Lillie and Juhn concluded that the asymmetry of the individual feather is a function of rates of growth in the two sides of the feather germ "at some time, or throughout development." Their interest was largely in the pigmentation configurations, and in certain definite instances they were able to observe asymmetries in the pigmentation pattern of the germ which were in close correspondence with configurations exhibited by the definitive feather. They also pointed out the dependence of degree of asymmetry upon position of the feather within the tract. Their observation of germinal differentials which were in correspondence with reverse asymmetries on opposite sides of the body are of particular interest. Lillie (1931) applied the principles set forth in the later paper by Lillie and Juhn to explanation of the partial and completely bilateral gynandromorphs found in birds.

Juhn and Fraps (1934) have noted that the symmetry relations along transverse rows of the breast tracts, as measured by lengths of barbs opposed at the shaft, followed an orderly distribution with respect to a secondary axis lying approximately at the sixth row, counting laterally from the mid-line of the bird. The increasing degree of asymmetry with reference to this secondary axis was in accord with previous observations of Juhn (unpublished data) that pigmentation patterns limited to one vane-half of the feathers of the breast tract showed the relation of mirror images within each tract, and that the degree of asymmetry in such patterns was of increasing order toward the lateral margins of the tract.

The study of Holmes (1935) demonstrated that there is a definite time and space order in the origin of follicles composing the individual plumage tracts. In the breast tracts particularly, the relation of this order to asymmetry in the adult plumage is clear: The first row of follicles to arise is parallel with the anteroposterior axis of the bird's body and is the sixth row of the definitive tract in lateral order from the mid-ventral line of the body. After the first row (No. 6 of the transverse sequence of the completed tract) has been laid down,

anteroposterior rows lateral to this arise in order. Since the degree of asymmetry of the adult plumage increases with distance from the No. 6 row, we may conclude that the degree of asymmetry corresponds with the order of origin in time of the rows composing the tract.

On the foregoing grounds it seems probable that the asymmetry of the individual feather follicle is a function of its position in the plumage tract in a much more exact sense than has been shown thus far. If this is true, methods other than those used in previous analyses are required. We have considered methods in the first paper of this series (Juhn and Fraps, 1936), and in the present paper we apply several of the described procedures to further analysis of asymmetry in the breast tracts. Our results furnish additional evidence for reversal of asymmetry in transverse sequences of the breast tract at or near the sixth position in mediolateral order. We have been able to show also, however, that not all of the characteristics of the individual feather in these tracts are referable to this axis. These results are of considerable theoretical interest, and in the third part of this paper we discuss briefly the probable nature of embryological processes involved in determination of properties within the plumage tract.

## I. Origin and Distribution of Follicles in the Breast Tracts

A brief statement of the observations of Holmes on the origin of follicles in the breast tracts will serve also to make clear the general arrangement of the individual follicles in the adult. The breast tracts are illustrated in text Figure 1 (from Holmes, 1935). *A–P* represents the anteroposterior axis of the bird. The row of black disks (1, 2, 3 . . . 26) represents the follicles of primary origin in the embryo, and is the sixth row in mediolateral order after completion of the tract. The follicles between the arrows are, according to Holmes, of approximately simultaneous origin in the embryo. Those anterior and posterior to the bases of the arrows arise later in time. After formation of the follicles composing the primary row, follicles arise to the right and left of it. The other rows composing the tract arise in order of distance from the row of origin in the tracts.

The individual follicles of the successive rows arise always at a point corresponding to the apex of an approximately isosceles triangle, the base of which is the line connecting two adjacent follicles of the previously formed anteroposterior row. The result of this order of origin is that the follicles of the completed tract represent a "herringbone" arrangement, and the lines connecting a series of follicles from the boundaries to the sixth row of the tracts are diagonals rather than right-angular co-ordinates.

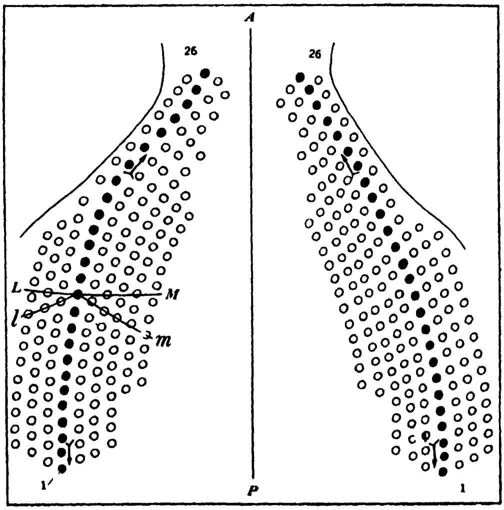


FIG. 1.—Order of origin of follicles in the breast tracts. *AP*, the anteroposterior axis (ventral) of the bird's body. The follicles represented as black disks and inclosed between the bases of arrows are of approximately simultaneous origin. Rows to right and left originate successively. The "true" transverse co-ordinate of the breast tract falls approximately along the line, *LM;* the actual transverse co-ordinate followed in experimentally ordered series is indicated by *lm*. (Adapted from Holmes, *Am. Jr. Anat.*, 56 [1935]: 513–37.)

In this paper we shall refer to a *transverse* sequence of follicles in the breast tract as the follicles along such diagonals, as *ml*, text Figure 1. The "true" transverse co-ordinate of the tract would lie along the line *ML*. We must take this displacement of lateralmost follicles from the true co-ordinates of the tract into account in the evaluation of certain results; this is particularly true of those properties of individual follicles which show also variation in antero-

posterior order, e.g., axial growth-rates. Properties of individual follicles of the transverse sequence which show reversal at the No. 6 position are not, however, materially different along the true transverse co-ordinates and the diagonal co-ordinates actually used in practice.

## II. Order of Distribution of Plumage Properties in the Transverse Sequence of the Breast Tracts

We are concerned here primarily with the distribution of properties in the breast tracts at approximately right angles to the anteroposterior axis of the tract (*ml* or *ML*, text Fig. 1). It should be clearly understood, however, that certain properties of the individual follicles (and feathers) of the tracts exhibit well-defined differences with respect to the anteroposterior axes. Juhn, Faulkner, and Gustavson (1931) have described at least two of these ordered functions: rate of growth of feathers and length to which regenerated feathers grow. Both these properties increase in anteroposterior direction. The fact that changes in rates and properties of the individual follicles vary through an area with two well-defined coordinates gives the breast tracts their primary *field* characteristics.

The properties of the nine follicles composing a transverse sequence of the breast tracts vary in three modes, which are conveniently distinguished as continuous, discontinuous, and polyphasic changes. A property which changes in the same direction from medial to lateral limits of the breast tracts, e.g., length of regenerated feather, will be referred to as a *continuous function* of the transverse co-ordinate of the tract. Properties which show definite reversal of direction of change (usually at or near the sixth follicle of the transverse sequence) will be denoted *discontinuous functions* of the transverse co-ordinates. *Polyphasic functions* describe properties which appear to involve both continuous and discontinuous functions along the transverse co-ordinate.

The follicles of the transverse sequence will be referred to always in mediolateral order as follicles 1 (nearest the ventral mid-line of the bird) to 9 (at the lateral boundary of the breast tracts).

### A. CONTINUOUS FUNCTIONS OF THE TRANSVERSE CO-ORDINATES

At least one property of the nine feather follicles composing a transverse sequence (*ml* of text Fig. 1) exhibits a continuous change

in mediolateral order through the tract in the Brown Leghorn capon, to which the present discussion is limited. This is the length to which regenerated feathers grow. Curves representing the average length of regenerated feathers from two adjacent rows are shown in text Figure 2*B*. Mediolateral positions are indicated by 1–9 on the common abscissas of Figure 2 (*A* and *B*); average lengths of the regenerated feathers are given in millimeters. The continuous increase in length of feathers is unbroken through the region of the axis of symmetry of the tracts, position No. 6. The curves differ somewhat in left and right tracts, but the general relations are well defined and cover a sufficient range of differences to eliminate the possibility that lengths of feathers along the true transverse co-ordinate (*ML* of text Fig. 1) might show a different relation. Since the length of completely regenerated feathers increases on all rows in anteroposterior order, the length of feathers on rows lateral to the sixth row of our curves are, in fact, somewhat less on the true transverse co-ordinate. Taking No. 6 as fixed, the net effect of this correction would be to flatten the curves slightly from positions 6 to 9 and to steepen the limb from positions 1 to 6.

A second set of lengths are plotted in text Figure 8. The plotted values represent average length and weight of feathers from six transverse sequences. Positions in the mediolateral sequence are given as abscissas; lengths in millimeters, by the left-hand ordinate. The relation of length of feather to position on the transverse co-ordinate is very similar to that shown in the curves of text Figure 2*B*. Incidentally, we might call attention to the marked relation between average length and average weight of feathers from the No. 1 to No. 6 positions of the transverse sequence, in contrast with the definite change in this relation shown by feathers from the 6 to 9 positions.

The individual measurements upon which the curves of text Figure 8 are based are given in Table I, together with weights of the same feathers. The feathers from corresponding positions are arranged in the usual mediolateral order, 1–9. The several sequences are from three birds, Nos. 1, 2, and 3, in the table. Lengths of feathers (millimeters) on two transverse sequences are given under *a* and *p* for bird 1. The number of follicles separating the sequences is not recorded, but row *a* was probably considerably anterior to

row *p*. The figures for birds 2 and 3 represent averages for adjacent feathers from the same transverse position. While there is some degree of variation in the order of changing length with position, the results are of much the same order. It is to be noted particularly that significant increases in length occur from sixth to ninth positions on all transverse sequences.

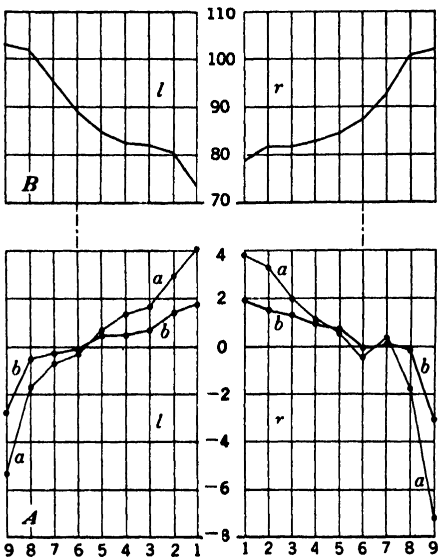


FIG. 2.—Graph *A*: differences in lengths of barbs in opposite vane-halves of feathers on 2 transverse sequences of the breast tracts. The figures at the base of the graph represent order of follicles from ventral mid-line of the bird's body. Ordinates, differences in millimeters, opposite vane-halves. Curves *a*, maximum differences in barb lengths; curves *b*, differences in barb lengths over the centimeters of greatest difference. Graph *B* gives length of the same feathers used in calculating the curves of graph *A*; ordinates, lengths in millimeters. Left and right sides of body: *l* and *r*.

The length of regenerated feathers is certainly not referable to the secondary axis of symmetry in the breast tracts of the Brown Leghorn capon. We have thus far been unable to be certain that any other property of the individual follicles shows so complete an independence of the axis of asymmetry (reversal at No. 6 position) as does this particular property. The length of a regenerated feather is a definite result of growth characteristics of the individual follicle (not necessarily growth-rates), and as such we conclude that it

measures a regenerative function of position with reference to the ventral mid-line of the bird's body.

We shall show later that rate of growth is not entirely correlated in the transverse sequence with length to which the individual feather grows. Inasmuch as the total length of the regenerated feather must, however, represent growth, we need only refer this property to longer period of growth, differences in growth-rates, or to combinations of these which may or may not in themselves be referable to other axes of the tract.

TABLE I

WEIGHTS AND LENGTHS OF FEATHERS FROM TRANSVERSE BREAST SEQUENCES

| Position No. | Bird 1: *a* | | Bird 1: *p* | | Bird 2: *a*, *b* | | Bird 3: *a*, *b* | | Averages | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mg. | Mm. | Mg. | Mm. | Mg. | Mm. | Mg. | Mm. | Mg. | Mm. |
| 1 | 41.4 | 75.0 | 39.5 | 83.5 | 28.3 | 77.0 | 35.9 | 75.3 | 34.9 | 77.2 |
| 2 | 40.5 | 84.0 | 39.4 | 84.0 | 30.9 | 77.0 | 37.6 | 76.8 | 36.1 | 79.2 |
| 3 | 39.7 | 85.0 | 41.4 | 85.0 | 32.5 | 78.5 | 38.3 | 76.5 | 37.1 | 80.0 |
| 4 | 41.2 | 85.5 | 40.6 | 87.0 | 34.0 | 79.0 | 40.0 | 79.8 | 38.3 | 81.7 |
| 5 | 43.5 | 89.5 | 42.0 | 91.5 | 37.3 | 83.5 | 41.9 | 82.3 | 40.6 | 85.4 |
| 6 | 44.9 | 91.5 | 46.5 | 94.0 | 41.6 | 84.5 | 45.1 | 87.0 | 44.1 | 88.1 |
| 7 | ...... | ...... | 44.1 | 95.0 | 41.4 | 93.8 | 47.8 | 93.8 | 44.5 | 94.0 |
| 8 | 47.7 | 94.0 | 45.0 | 100.0 | 41.4 | 99.0 | 48.0 | 99.8 | 45.3 | 96.9 |
| 9 | 44.5 | 94.0 | 45.2 | 101.0 | 40.0 | 97.8 | ...... | ...... | 42.4 | 97.6 |

Juhn, Faulkner, and Gustavson (1931) concluded that the rate of growth of regenerated feathers increased in mediolateral order from the ventral mid-line. Their measurements were made over relatively great intervals of time, and it is difficult to interpret their conclusions in terms of actual rates, and particularly rates which refer to more or less comparable periods in the regenerative cycle. A further analysis of growth-rates with reference to the transverse sequences of the breast tracts has shown that the maximum rate of growth must be defined for homologous phases in the regenerative cycle rather than as "averages" for relatively prolonged periods. These findings (unpublished data of Juhn) are probably of significance also in connection with the difficulties in interpretation of plumage response in breast tracts to intramuscular injection of

oestrone recorded by Greenwood and Blyth (1935*a*). We take up these growth-rate relations in later pages.

### B. DISCONTINUOUS FUNCTIONS OF THE TRANSVERSE CO-ORDINATES

By "discontinuous functions" we refer to those properties of the individual follicle which show a definite reversal of asymmetry or sign at or near the No. 6 position of the transverse sequence. Such functions are "discontinuous" with respect to the mediolateral co-ordinate of the breast tracts, and "reversal" in any relation or property involving opposite vane-halves occurs as a change in sign if we relate magnitudes describing the vane-halves uniformly for all feathers of the transverse sequence.

#### 1. LENGTH OF OPPOSED BARBS IN BREAST FEATHERS

In a brief note by the present authors (Juhn and Fraps, 1934), we showed that *differences* in the length of simultaneously completed barbs in the series of breast feathers composing a transverse sequence were least at or near the No. 6 position, and that differences in length of opposed barbs increased in order of progression toward lateral margins of the tracts. These results have since been considerably extended. We give in text Figure 2*A* curves for two length indexes of barbs in opposite vane-halves. The numbers 1–9 from the center of the graph represent transverse positions in mediolateral order from the ventral mid-line of the bird; ordinates represent differences in length of barbs in millimeters. Curve *a* in each graph is the maximum difference in length of completed barbs; curve *b* represents average differences over the centimeter of greatest difference in length of simultaneously completed barbs. The two curves show that least differences are found in the neighborhood of the No. 6 axis. This is more definitely brought out in the left breast tract than in the right, but in neither instance is there any doubt concerning the general trend of the curves from medial to lateral margins of the tract.

It should be pointed out that differences in lengths of simultaneously completed barbs are plotted continuously across the tract; a value of 0, therefore, represents no difference in the two vane-halves of the feather and is an index of approximate symmetry with respect

to the particular index chosen. Negative differences become positive, of course, if we reverse the order of subtraction in vane-halves at (or approximately at) the Nŏ. 6 position in mediolateral progression. The usual relation of reversed symmetry with reference to the No. 6 position is then evident by the upward curvature of both limbs of the curve from the No. 6 position.

In recording measurements of length of simultaneously completed barbs at intervals from apex to base of the shaft, we have observed that there is a common tendency for differences in lengths to show a definite order of change in at least those feathers near No. 6 positions. It is difficult to interpret in terms of asymmetry either maximum differences in barb lengths or averages of differences for any considerable apicobasal section of these "least asymmetric" feathers. This situation is made clear in text Figure 3(*A* and *B*), which shows the differences in length of opposed barbs at 5-mm. intervals from apex of the shaft through 80-mm. shaft lengths. The curves of Figure 3*A* are for feathers from the No. 6 positions; curves of Figure 3*B* are for feathers from the No. 7 positions. The letters *a* and *b* indicate adjacent anterior and posterior feathers.

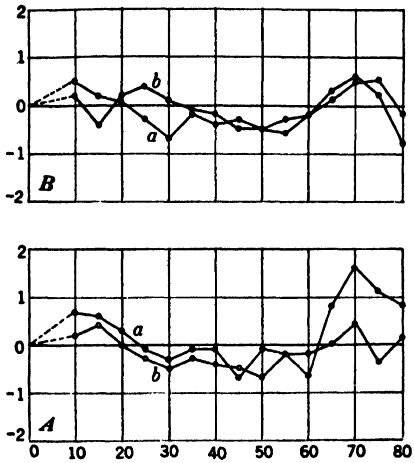


FIG. 3.—Reversal in lengths of barbs at completion in opposite vane-halves. Abscissas, distances from apex of feather in millimeters. Ordinates, differences in barb lengths in millimeters. Graph *A*, feathers from the No. 6 position; graph *B*, feathers from the No. 7 position. The small letters, *a* and *b*, refer to individual feathers from different anteroposterior sequences.

It will be observed that differences in barb lengths are positive through approximately 20 mm., after which differences become negative, and remain so, to about 60 mm. At or near the 60-mm. shaft level there occurs a second reversal of sign. It is probably significant that reversals of this order are more marked in feathers near the

sixth position. While the asymmetry of an individual feather, as based upon an "average" index, may be close to zero, it is obvious that an index based upon differences in lengths of barbs at chosen intervals along the shaft may give either positive or negative values for the "asymmetry" of the feather. For these reasons we have considered the curves given in text Figure 2*A* to be simply "indexes," which, while indicating a real reversal of asymmetry at or near the No. 6 position, are not of great value in other respects.

### 2. ASYMMETRIC MAGNITUDES DETERMINED BY C-ISOCHRONE ANALYSIS

Methods for the application of the c-isochrone to the completely regenerated feather are described in the first paper of this series. We have applied these methods (in part) to three feathers of a single transverse sequence. The results given here are concerned mainly with determination of relations in the opposite vane-halves. The feathers used for analyses are treated individually, and we shall attempt to show only that the various determined magnitudes agree in locating the axis of reversal of asymmetry in the neighborhood of the sixth position of the transverse sequences. A complete analysis of the breast tracts of the Brown Leghorn will require treatment of more comprehensive series than we have yet attempted, and the present results are necessarily of a preliminary nature. It will nevertheless be clear that reversal of signs in magnitudes measuring relative asymmetry occurs definitely in feathers 3 and 9, while in feather 6 the same measures show least values. The quantitative formulation of similar relations for complete sequences will be the subject of a later analysis.

The three feathers for which we present data are from a Brown Leghorn capon. We have some evidence that asymmetry differentials are less marked in the female; whether these differences are due to individual variations rather than to differences in sex has not been determined. A few preliminary results on breast tracts of the Barred Rock show also rather marked deviations from the results given here. For these reasons we wish to make it clear that the conclusions drawn from this analysis are limited to the Brown Leghorn capon.

*a) Cumulative barb numbers.*—The derivation of cumulative barb numbers and their significance are taken up in the first paper of this series.

The marginal c-isochrone determinations for feathers 3, 6, and 9 of a transverse sequence (feathers reproduced in Pl. I) are given in Table II. Column *c* is the number of successive c-isochrones; *d* is the distance in millimeters from apex of the feather to successive c-isochrones; *l* and *r* refer to cumulative numbers of barb apexes defined in left and right vane-halves. It is evident from the figures presented

TABLE II

MARGINAL BARB NUMBERS, FEATHERS 3, 6, AND 9 OF TRANSVERSE BREAST SEQUENCE

| *c* | Feather 3 | | | Feather 6 | | | Feather 9 | | |
|---|---|---|---|---|---|---|---|---|---|
| | *d* | *l* | *r* | *d* | *l* | *r* | *d* | *l* | *r* |
| 1...... | ....... | 25.0 | 15 0 | ....... | 8.5 | 7.2 | ....... | 3.0 | 11.2 |
| 2...... | 0.64 | 29.7 | 23.5 | 0.45 | 17.0 | 14 8 | 0.64 | 9.0 | 22 6 |
| 3...... | 1.28 | 32.7 | 29.2 | 1.12 | 28.0 | 25.0 | 1.57 | 19.4 | 33.5 |
| 4...... | 1.92 | 36.4 | 34.2 | 1.95 | 37.2 | 34.6 | 2.69 | 31.0 | 43.0 |
| 5...... | 2.88 | 41.8 | 42.7 | 3.36 | 47.6 | 45.2 | 4.32 | 44.0 | 51.0 |
| 6...... | 4.16 | 49.0 | 52.0 | 4.70 | 55.0 | 52.0 | 7.08 | 57.0 | 60.8 |
| 7...... | 5.77 | 56.6 | 59.0 | 5.82 | 60.3 | 57 0 | 9.79 | 69.7 | 71.8 |
| 8...... | 7.69 | 64.8 | 68.0 | 7.97 | 70.7 | 66.2 | 14 34 | 87.0 | 86.0 |
| 9...... | 10.24 | 75.0 | 77.2 | 10.88 | 81.8 | 77.0 | 18.01 | 100.2 | 99.0 |
| 10...... | 13.77 | 88.0 | 92.0 | 13.50 | 90.7 | 86.7 | 22.34 | 116.0 | 112.0 |
| 11...... | 17.92 | 101.2 | 107.0 | 16.38 | 100.0 | 95.6 | 27.42 | 131.0 | 126.0 |
| 12...... | 22.72 | 116.0 | 122.0 | 19.52 | 110.0 | 105.8 | 32.70 | 147.0 | 138 5 |
| 13...... | 27.74 | 128 0 | 135.0 | 23.42 | 121.5 | 118.0 | 39.68 | 166.0 | 156.0 |
| 14...... | 31.55 | ....... | ....... | 27.04 | 132.0 | 127.2 | ....... | ....... | ....... |
| 15...... | ....... | ....... | ....... | 30.82 | 143.0 | 138.0 | ....... | ....... | ....... |
| 16...... | ....... | ....... | ....... | 34.40 | 152.0 | 147.5 | ....... | ....... | ....... |
| 17...... | ....... | ....... | ....... | 37.44 | 160.0 | 156.0 | ....... | ....... | ....... |

in this table that feather 6 shows least differences in opposite vane-halves.

The data of Table II applying to feather 3 are represented by the curves $N_m$ of text Figure 4; the corresponding data for feather 9 are given in text Figure 5. Abscissas in both figures represent distances in millimeters from apex of the feathers; marginal barb numbers, $N_m$, are represented by ordinates.

Marginal barb numbers define the cumulative numbers of barb apexes "determined" at given axial levels of growth (Juhn and Fraps, 1936). Similarly, shaft barb numbers define the number of barb bases "completed" at definite axial levels of growth. Shaft barb numbers are given in Table III, and the data are graphed as

curves $N_s$ in text Figures 4 and 5 for corresponding marginal barb numbers and abscissas (feather 3 in Fig. 4; feather 9 in Fig. 5).

Marginal barb numbers increase with relatively great rapidity near the apex of both feathers. During the initial stages of barb formation, marginal numbers are higher in the left vane-half of feather 3 (text Fig. 4); in feather 9 (text Fig. 5) the reverse is true. In both feathers 3 and 9 the initial relations are subsequently reversed with respect to opposite vane-halves.

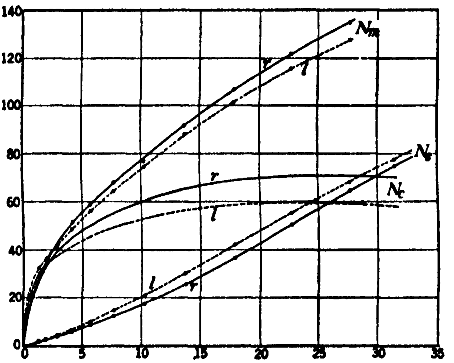


FIG. 4.—Asymmetry relations defined by c-isochrone constructions in feather 3 of transverse breast sequence (Pl. I). Abscissas, shaft distances in millimeters from apexes of the primary barbs; ordinates, barb numbers. Curves $N_m$, marginal barb numbers; curves $N_s$, shaft barb numbers; curves $N_c$, barbs on successive isochrones ($N_c = N_m - N_s$). Broken lines ($l$), left vane-half; solid lines ($r$), right vane-half.

These relations apparently introduce in a quite different form the reversal of asymmetry which we have already observed to be characteristic of difference in lengths of simultaneously completed barbs.

It is obvious that the initial stages of barb formation (curves $N_m$) represent relations which are the reverse of relations obtaining in formation of the main vane structure, i.e., from approximately 5 mm. basally. The initial phase of ridge formation is characterized by simultaneous, or very rapid, formation of ridges in proportion to axial growth of the germ (Fraps and Juhn, 1936). This initial rapid rate of barb formation represents the formation of ridges in *dorso-ventral order* in cells already formed in the wall of the feather germ. After completion of collar-limb complements, barbs are laid down serially at the ventral triangle of the germ. The formation of ridges in these differing modes is probably associated with the *apparent* reversal of relations so clearly brought out in the curves of text Figures 4 and 5. If this is true, it is highly probable that both initial formation of ridges and serial formation of ridges through the main

vane structure are referable to the same fundamental measure of asymmetry (cf. text Fig. 6).

The curves for shaft barb numbers, $N_s$, as measured by cumulative numbers of barb bases at successive c-isochrones, show similar relations. The *absolute* relations are, however, the reverse of those shown by curves for cumulative marginal barb numbers. This is clearly brought out if we compare the four curves beyond the first 7 mm. of shaft length. Cumulative shaft numbers (feather 3) are

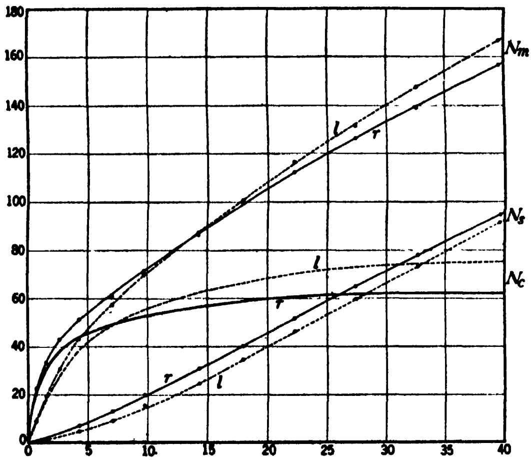


FIG. 5.—Asymmetry relations in feather 9 of transverse breast sequence. Abscissas, shaft distances in millimeters from apexes of primary barbs; ordinates, barb numbers. Curves $N_m$, marginal numbers; curves $N_s$, shaft numbers; curves $N_c$, barbs on successive c-isochrones. Broken lines ($l$), left vane-half; solid lines ($r$), right vane-half. Note the reversal of all relations shown in text Figure 4.

higher in the left vane-half; the corresponding marginal numbers are lower in this vane-half. The curves for feather 9, text Figure 5, show exactly the opposite relations in every respect beyond ca. 12 mm.

If we take some level following establishment of the complete barb complement, say at about the 30-mm. level, the left collar limb of feather 9 (text Fig. 5) is laying down barbs at a more rapid relative rate. These barbs are completed, however, at a slower relative rate. Again, feather 3 shows opposite or "reverse" relations in every detail.

The figures of Table II show that feather 6 is subject to rela-

tively small differences in cumulative barb numbers at the margin (barbs laid down). Differences in relative numbers of barbs completed are even less, as is shown in Table III.

It is not necessary to attempt an interpretation of these results further than to point out that actual numbers of barb apexes laid down, or of barbs completed, represent definite "events" in the developmental process. If the numbers which measure these events

TABLE III

SHAFT BARB NUMBERS, FEATHERS 3, 6, AND 9 OF TRANSVERSE BREAST SEQUENCE

| *c* | Feather 3 | | | Feather 6 | | | Feather 9 | | |
|---|---|---|---|---|---|---|---|---|---|
| | *d* | *l* | *r* | *d* | *l* | *r* | *d* | *l* | *r* |
| 1 | ...... | ...... | ...... | ...... | ...... | ...... | ...... | ...... | ...... |
| 2 | 0.64 | ...... | ...... | 0.45 | ...... | ...... | 0.64 | ...... | ...... |
| 3 | 1.28 | 2.0 | 1.5 | 1.12 | ...... | ...... | 1.57 | 1.0 | 1.0 |
| 4 | 1.92 | 2.7 | 2.4 | 1.95 | 0.7 | 0.7 | 2.69 | 2.3 | 3.0 |
| 5 | 2.88 | 4.3 | 3.8 | 3.36 | 3.0 | 3.4 | 4.32 | 4.7 | 7.0 |
| 6 | 4.16 | 6.6 | 5.7 | 4.70 | 5.2 | 6.0 | 7.08 | 9.0 | 13.0 |
| 7 | 5.77 | 10.0 | 8.3 | 5.82 | 7.0 | 8.2 | 9.79 | 15.1 | 19.8 |
| 8 | 7.69 | 14.7 | 12.2 | 7.97 | 11.4 | 12.5 | 14.34 | 24.5 | 30.7 |
| 9 | 10.24 | 21.0 | 17.6 | 10.88 | 18.3 | 19.0 | 18.01 | 34.4 | 40.0 |
| 10 | 13.77 | 30.5 | 25.7 | 13.50 | 25.0 | 25.3 | 22.34 | 46.0 | 51.3 |
| 11 | 17.92 | 42.3 | 37.0 | 16.38 | 32.8 | 32.3 | 27.42 | 59.3 | 64.7 |
| 12 | 22.72 | 56.0 | 51.0 | 19.52 | 41.2 | 41.0 | 32.70 | 73.0 | 77.5 |
| 13 | 27.74 | 68.5 | 65.0 | 23.42 | 52.7 | 52.0 | 39.68 | 91.0 | 94.0 |
| 14 | 31.55 | 78.0 | 75.5 | 27.04 | 62.7 | 62.0 | ...... | ...... | ...... |
| 15 | ...... | ...... | ...... | 30.82 | 73.0 | 73.0 | ...... | ...... | ...... |
| 16 | ...... | ...... | ...... | 34.40 | 84.0 | 83.5 | ...... | ...... | ...... |
| 17 | ...... | ...... | ...... | 37.44 | 93.0 | 92.0 | ...... | ...... | ...... |

show differentials in the two vane-halves of an individual feather, and a definite reversal of these differentials in feathers on opposite sides of the No. 6 position, the axis of symmetry, we are clearly dealing with a reversal of asymmetry which most probably occurs in the neighborhood of the No. 6 feather of the transverse sequence.

Curves representing the marginal barb numbers are possibly a more direct measure of reversal of asymmetry than are curves for shaft barb numbers. It is to be noted that the differences exhibited by the two sets of curves are in every case more marked for marginal barb numbers than for barb numbers at the shaft intercepts.

*b*) *Barbs on c-isochrones.*—Table IV gives the number of barbs on successive c-isochrones applied to feathers 3, 6, and 9 at the distances indicated from the apex of each feather. The number of barbs thus defined by c-isochrones for the feather from the third row are shown in text Figure 4, curves $N_c$, *r* and *l*, representing right and left vane-halves. The corresponding curves for feather 9 are shown in text Figure 5. The number of barbs on successive c-isochrones in the

TABLE IV

BARBS ON C-ISOCHRONES, FEATHERS 3, 6, AND 9 OF TRANSVERSE BREAST SEQUENCE

| *c* | Feather 3 | | | Feather 6 | | | Feather 9 | | |
|---|---|---|---|---|---|---|---|---|---|
| | *d* | *l* | *r* | *d* | *l* | *r* | *d* | *l* | *r* |
| 1...... | ...... | ...... | ...... | ...... | ...... | ...... | ...... | ...... | ...... |
| 2...... | 0.64 | ...... | ...... | 0.45 | ...... | ...... | 0.64 | ...... | ...... |
| 3...... | 1.28 | 30.7 | 27.7 | 1.12 | ...... | ...... | 1.57 | 18.4 | 32.5 |
| 4...... | 1.92 | 33.7 | 31.8 | 1.95 | 36.5 | 33.9 | 2.69 | 28.7 | 40.0 |
| 5...... | 2.88 | 37.5 | 38.9 | 3.36 | 44.6 | 41.8 | 4.32 | 39.3 | 44.0 |
| 6...... | 4.16 | 42.4 | 46.3 | 4.70 | 49.8 | 46.0 | 7.08 | 48.0 | 47.8 |
| 7...... | 5.77 | 46.6 | 50.7 | 5.82 | 53.3 | 48.8 | 9.79 | 54.6 | 52.0 |
| 8...... | 7.69 | 50.1 | 55.8 | 7.97 | 59.3 | 53.7 | 14.34 | 62.5 | 55.3 |
| 9...... | 10.24 | 54.0 | 59.6 | 10.88 | 63.5 | 58.0 | 18.01 | 65.8 | 59.0 |
| 10...... | 13.77 | 57.5 | 66.3 | 13.50 | 65.7 | 61.4 | 22.34 | 70.0 | 60.7 |
| 11...... | 17.92 | 58.9 | 70.0 | 16.38 | 67.2 | 63.3 | 27.42 | 71.7 | 61.3 |
| 12...... | 22.72 | 60.0 | 71.0 | 19.52 | 68.8 | 64.8 | 32.70 | 74.0 | 61.0 |
| 13...... | 27.74 | 59.5 | 70.0 | 23.42 | 68.8 | 66.0 | 39.68 | 75.0 | 62.0 |
| 14...... | 31.55 | ...... | ...... | 27.04 | 69.3 | 65.2 | ...... | ...... | ...... |
| 15...... | ...... | ...... | ...... | 30.82 | 70.0 | 65.0 | ...... | ...... | ...... |
| 16...... | ...... | ...... | ...... | 34.40 | 68.0 | 64.0 | ...... | ...... | ...... |
| 17...... | ...... | ...... | ...... | 37.44 | 67.0 | 64.0 | ...... | ...... | ...... |

right vane-half increases very rapidly during the initial stages of barb formation; the curves then fall for both vane-halves, and the number of barbs in the right collar limb becomes in the instance of feather 9, text Figure 5, less than the number in the left collar limb at about 7 mm. axial growth of the germ. The number of barbs in the left collar complement remains definitely above the number in the right collar limb after the curves have crossed.

The rate at which the right collar complement, feather 9, text Figure 5, is established is much greater than the rate at which the barb complement of the left collar limb is completed. Nevertheless,

during the formation of the main part of the vane of feather 9, the number of barbs in the left collar is considerably greater than in the right collar limb.

Curves for the number of barbs in the collar limbs of the third feather in the transverse row (text Fig. 4) are the reverse of those shown by feather 9, i.e., the number of barbs in the left collar limb increases more rapidly during the initial stages of axial growth, and at later stages the right collar limb comes to have the greater number of barbs.

Table IV shows that the number of barbs in the collar of the feather from the sixth position is almost perfectly symmetrical on corresponding c-isochrones. This is, in fact, evident from the data shown in Tables II and III, since marginal and shaft barb numbers on successive isochrones are almost uniform in the two vane-halves.

It is not certain just what significance should be attached to the number of barbs on opposed c-isochrones, since we do not know the exact relations which obtain between number of barbs, size of barbs, and possible changes in the absolute length of collar between the ventral triangle and the shaft primordium. But it is certain, nevertheless, that we are dealing here also with well-defined phases of asymmetry. This is further borne out by the remarkable manner in which reversal of the relations of asymmetry in feathers 3 and 9 is shown in the initial stages of growth (i.e., the first 3 or 4 mm. of axial growth) as well as in the later stages of more or less complete barb complements. The reversal of symmetry relations is definitely shown at all comparable levels of feathers 3 and 9.

*c*) *Relative ratios in marginal barb numbers.*—We have shown elsewhere (Juhn and Fraps, 1936) that the barb numbers in opposite vane-halves may be compared through the main vane structure according to the formula $y = bx + a$. If we plot the number of barbs formed at successive isochrones in the opposite vane-halves as abscissas and ordinates, the data describe a straight line for opposite vane-halves through at least the main portion of the vane.[2] In text Figure 6 we give the data for feathers 3 and 9, marginal intercepts only, thus plotted (data from Table II). Abscissas represent the

[2] We use this formula in the present connection purely as a mode of description. See Juhn and Fraps, 1936.

marginal barb numbers in left vane-halves; ordinates represent marginal barb numbers at corresponding levels in the right vane-half.

It is evident from Table II that the relative ratio of marginal barb numbers is practically unity in the vane-halves of feather 6; from the same table it is evident that marginal numbers increase in feather 9 more rapidly on the left side, while in feather 3 the more rapid increments are on the right side. We have determined by graphic methods the slope of the line through the region of constant

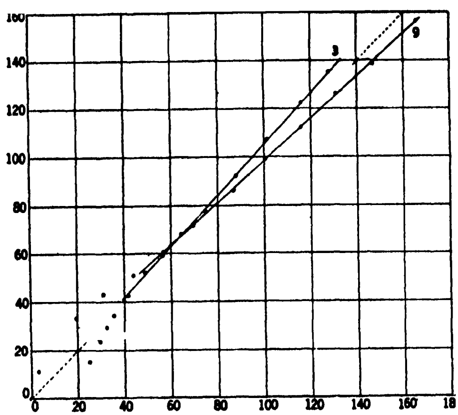


FIG. 6.—Relative ratios in marginal barb numbers, feathers 3 and 9 of transverse breast sequence. Abscissas represent (for each feather) barb numbers in left vane-half; ordinates, barb numbers in right vane-half at corresponding c-isochrone intercepts. In a perfectly symmetrical feather, barb numbers would be equal in left and right vane-halves at all points; the slope of the line through these points would be unity.

relative marginal ratios for each of the three feathers. The equations are as follows:

Feather 3: $y=1.07x-2.$

Feather 6: $y=0.98x-2.$

Feather 9: $y=0.87x+11.$

A value of $b=1$ in these results would mean, of course, that no differentials are present. In feather 3 the relative marginal ratio is practically constant between barbs 50 and 130. In the longer feather (No. 9) the ratio is approximately constant from the fiftieth barb through formation of the 165th barb. We have not given the curve

for feather 6; it is evident from the formula that the differences are very small.

It should be emphasized that the data plotted in text Figure 6 represent relative ratios in a definite "serial" process, i.e., the rates at which apical barb loci are "defined" as determined by application of the c-isochrone. The straight lines drawn through the determined points show beyond doubt that the given formula adequately describes the relation between these magnitudes in the opposite vane-halves of the feather. It might be objected that the differences actually shown by these feathers are small; but it should be realized that all asymmetries in the breast tracts are of relatively small degree, as compared, for example, with feathers of the wing tracts.

We cannot at present definitely evaluate the curves shown in text Figure 6 either in terms of growth or differentiation. But as we have attempted to emphasize in connection with all of the data presented here, the important relation is that a definite reversal of sign or magnitude characterizes feathers 3 and 9 in all c-isochrone measures. Relative ratios in marginal barb numbers are probably the most direct measure of this reversal that we have been able to obtain.

Application of the c-isochrone to the feathers of the transverse sequence dealt with here shows by all measures or "indexes" that feather 6 is fairly symmetrical, and that reversal of asymmetry in feathers 3 and 9 occurs in the order expected on the basis that the No. 6 position represents in fact the axis of symmetry in the tracts.

### 3. GROWTH-RATES OF REGENERATING FEATHERS ON TRANSVERSE CO-ORDINATES

We include these data here although as noted earlier the rate of growth of regenerating feathers on a transverse sequence must be compared for definite periods in the regenerative cycle.

Curves representing the growth increments of two transverse sequences (on opposite sides of the bird's body) are given in text Figure 7. The positions of feathers in mediolateral order are given at the base of the figure; ordinates represent simultaneous lengths of regenerating feathers, adjusted to a 5-mm. initial length in order to eliminate differences in times of emergence.

The topmost curves show, in both sequences, maximum lengths

in the region of the axis of symmetry; we cannot say, however, that there is a well-defined "peak" at the No. 6 position. Nor are growth increments uniformly greater in the region of the axis of symmetry: at approximately 20 mm. in length, maximum rate of growth appears to characterize positions medial to the No. 6 position.

Correction of these data by reference to the "true" transverse co-ordinates of the breast tracts would accentuate growth increments in the region of the axis of symmetry. The differentials represented are therefore to be taken as minimal for these particular sequences.

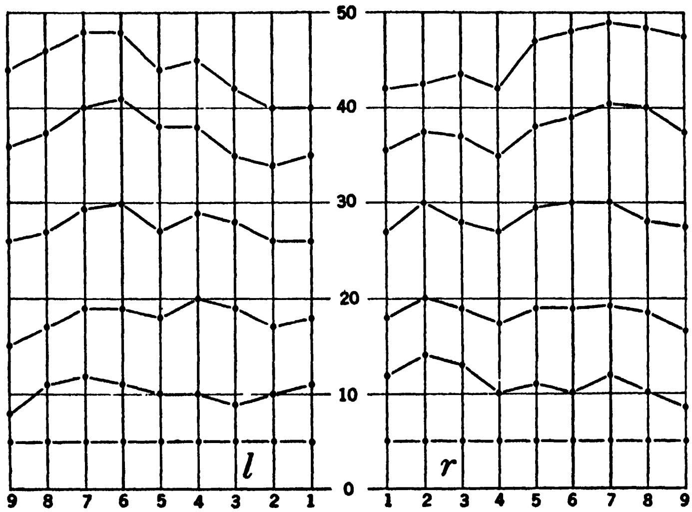


FIG. 7.—Growth increments in regenerating feathers of transverse sequences, breast tracts. Abscissas, mediolateral order of left and right sequences; ordinates, lengths of regenerating feathers. Each pair of curves (right and left tracts) represents the lengths of feathers at the same time, adjusted on basis that all feathers are 5 mm. in length at time of first determinations.

We are clearly dealing here with a complex situation—certainly one that cannot be reduced to the orderly relations describing total lengths of feathers on the transverse sequences or the relations of reversed asymmetry as measured by c-isochrone magnitudes. We shall accordingly treat these results with some reservations at this time.

C. POLYPHASIC FUNCTIONS OF THE TRANSVERSE CO-ORDINATES

The weights of regenerated feathers lying along transverse sequences show a striking discontinuity when compared with the data presented under the foregoing orders. Text Figure 8 represents average weights of feathers for six rows. The unbroken curve is the average length of regenerated feathers 1–9 for the same transverse sequences. As we have shown earlier, the length of feathers increases continuously across the tract. In feathers 1–6 of the transverse sequence, the total weight of the completely regenerated feather is almost directly proportional to the length of the feather. Weights from the sixth to the ninth feather, however, show the curve to break sharply; and feather 9 shows a definite decrease in total weight, although length of the completely regenerated feather is greatest for the sequence.

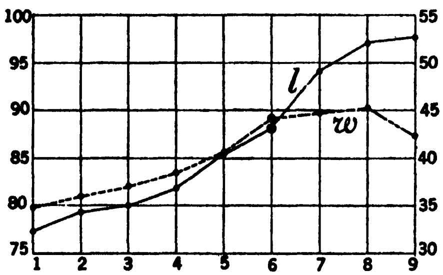


FIG. 8.—The relation between length and weight of regenerated feathers from six transverse sequences of the breast tracts. Abscissas, positions of follicles in mediolateral order; left ordinates, length of the regenerated feathers in millimeters; right ordinates, weight of the same feathers in milligrams. Curve *l*, length of feathers; curve *w*, weight of feathers.

If, as we have suggested earlier, the length of completely regenerated feathers may be treated as a continuous function, while those properties of feathers which show a true reversal of asymmetry are treated as discontinuous functions, we might interpret the data on weight of regenerated feathers to represent a partial relation between these two determinative functions. The exact character of this possible relation need not be discussed here. It should be emphasized that we use the term "polyphasic" to describe an actual incongruity of relation, and for the moment we need not attach too much significance to the term.

We summarize the experimental results under the following three headings, which are referable to the observations presented under the notation of continuous, discontinuous, and polyphasic functions:[3]

[3] According to Greenwood and Blyth (1935*b*), asymmetrical pigmentation reactions

1. Properties of the transverse sequences which change continuously through the tract in mediolateral order.
2. Properties of the transverse sequences which change continuously from feather 1 to feather 6, and show definite reversal from feather 6 to feather 9.
3. Properties of the transverse sequences which show a definite relation, direct or inverse, through one limb of the symmetry orders (feathers 1–6) but are not so related in the other limb of the order (feathers 6–9).

### III. THE DISTRIBUTION OF PLUMAGE PROPERTIES AS FIELD FUNCTIONS

The relations established in the first part of this paper involve, as a matter of course, the concept of gradients or fields. We are concerned here primarily with transverse co-ordinates, and particularly with the fact that the properties of individual follicles on these co-ordinates are referable to two distinct anteroposterior axes: we recognize therefore two "incongruous" transverse gradients.

Since we interpret the relations recorded in this paper as "gradient" or "field" relations, we shall begin with a statement of what we understand by these terms. We shall not attempt a critical examination of the many concepts of fields and gradients which have appeared in the literature. Discussions on these lines have come recently from Huxley (1935) and Weiss (1935).

The most comprehensive experimental analysis and theoretical development of relations implicit in either the gradient or the field concept is that of Child, and we wish to emphasize that we have used the term "field," rather than "gradient," in view of the two-dimensional gradient vectors of the plumage tracts. The analytic datum remains the gradient or physiological axis within the space of the

may be induced in the breast tracts independently of either of the transverse orders which we describe here. The distribution of asymmetries effected by microinjection of female hormone is referable to the site of injection as a center of diffusion, and Greenwood and Blyth conclude that the action of the hormone is "a direct one." If this is true, it is clear that reactions of such an order cannot involve directly or entirely the primary asymmetry differentials of the germ. The results described by Greenwood and Blyth bring up interesting questions concerning the mechanism of transport and distribution of the hormones, but these need not concern us further here.

field; and the gradient concept as this has been developed by Child is from this point of view the most representative characterization of the primary differentials composing the field that has yet appeared.

In the present discussion of fields we shall attempt to avoid two common implications. The first is that the *field* represents a "general" and more or less indefinite set of relations rather than exact and limited relations. If functions referable to a gradient or a field are exact, e.g., the properties of individual feather follicles composing a transverse sequence, it is logically necessary to assume that the field energy which determines (or has determined) those properties and their distribution must also be (or have been) equally precise in distribution and limited in specific action. This does not mean that the distribution of field energies or gradient differentials is an invariable relation, a point sufficiently evident in view of the ease with which gradient differentials are subject to modification.

The second implication in the concept of fields is that of "ultimate" explanatory values without due reference to the fundamental properties and characterization of the dynamic field particularly and in its simplest physical sense. That some primary distinction must be made between fields is sufficiently evident in the recognition of direct chemical action as a definite phase of "organizer" action, distinct from the dynamic rôle of the "individuation field" (Waddington and Schmidt, 1933). The transmissive (energy) nature of gradient (and *ipso facto* field) differentials of a *primary order* recognized earlier by Child (1928 *et ante*) is implicit in Waddington and Schmidt's distinction, and likewise in Huxley's (1935) "distance" and "contact" organizers.

### A. ENERGY AND RESIDUAL FIELDS

In a strictly dynamic sense, a field is an orderly distribution of energy in space. The distribution of the field energy is not homogeneous, i.e., it is not uniform at every point within the space of the field but is subject to differentials in *intensity* and in *direction;* it is these differentials which we evaluate, directly or indirectly, in defining a field.

A *point* in a purely dynamic field is completely defined by the in-

tensity and direction of the field energy at that point, and the *field* is completely defined in terms of the intensity and direction of the field energy at all points within the space of the field. In the classical representation of Faraday the (electromagnetic) field is conceived in terms of lines of force: the direction of the lines at a point is the direction of the field energy at that point, and the density of the lines represents the intensity of the field at that point. Biologically, of course, the problems presented by field energies are "unique" in two respects: specificity of energy and specificity in effect.

The distribution of forces within the space of a strictly physical field will be symmetrical with respect to defined loci ("centers," axes) only if the space of the field (or the distribution of bodies within the space of the field) is uniformly or symmetrically "permeable" to the field energy. "Distorted" or "asymmetrical" fields[4] are not, therefore, *a priori* evidence for a corresponding distortion or asymmetry in distribution of the same field energy as an inherent property of the energy itself. It is therefore necessary to distinguish in the morphogenetic process "apparent" and "real" energy fields.

1. A (persistent) dynamic field involves the presupposition of energy relations which are maintained with reference to centers or axes from which there are definite and exact decrements in the field energy. The experimentally imposed physiological gradients of Child (1924, 1928) are, in their simplest form, examples of dynamic field energies from this point of view.

2. A temporary dynamic field may determine once and for all a set of relations which remain constant subsequent to the *determinative* action of the field. The determined properties, or relations, will thenceforth not require the continued action of the primary energy field for the expression of differentials. We shall refer to the composite differential properties of any such "apparent" field as a *residual* field. Putting the relation a little differently, and with the properties of plumage tracts as example, we define a residual field as the order of differentials imposed by a dynamic field upon a series or

[4] The peculiar weight curves for feathers composing a transverse sequence may be significant in this connection, on the assumption that the "forces" referable to the No. 6 position must operate in a substrate with previously established heterogeneous properties, i.e., "lower" toward the No. 1 position, "higher" toward the No. 9 position.

aggregate of individual "points" or mechanisms, and thenceforth maintained independent of the morphogenetically effective energy.

Dynamic properties (e.g., growth-rates), may characterize residual fields. The existence of such properties is not, in itself, evidence that a dynamically effective causal energy is the "source" of differentials exhibited by the "points" or mechanisms composing the manifold or aggregate, e.g., the plumage tract. The dynamic phase in the morphogenetic process may, at least theoretically, be strictly limited to the *determination* of the properties of differing points or mechanisms within the original space of the field. Once determined, the several points or mechanisms must show differential potentialities or properties—"dynamic" or otherwise—which are field functions in a strictly residual sense.

If these propositions have been made clear, it follows that we have at least two distinct possibilities in the definition of all apparently dynamic fields in the organism. The first is that the field may represent a true energy field in the strict sense that differentials between points within the space of the field are dependent upon a continued or "persistent" distribution of a field energy. The second is that a temporary energy field may bring about definite determinations upon, or configurations within, masses (bodies, e.g., genes) within the space of the field. The initial transforming field energy need not, in this instance, be maintained; its dynamic function is in this sense strictly that of determination.[5] Differential properties, potentialities, or intensities exhibited by individual aggregates thus determined, compose residual fields in the limited sense that we apply that term to the plumage tracts.

### B. DISCRETE TERMS IN THE MORPHOGENETIC PROCESS

The ideal measure of a dynamic morphogenetic field, as of any "true" field, would be the absolute intensity of the field energy at a

[5] We use the term "determination" here strictly with reference to the properties of individual follicles described earlier; if these properties are, in fact, residuals referable to previously established (however temporary) dynamic field differentials, determination in the limited sense that we employ the term here refers to the specific *transformation* in the reactive substrate which shall thenceforth define or characterize the substrate (and its derivatives) independently of antecedent dynamic field energies. We might say, somewhat schematically, that residuals represent *determinations* and that differentials in the initial field energy represent *potentials*.

sufficient number of points within the space of the field to permit of its complete mathematical formulation. In the absence of so elegant a measure of even the simplest type of field or gradient differential in the organism, we can only determine the distribution of *properties* which represent either direct field variables *or residuals* of an assumed primary field energy.

Theoretically at least, the properties of the plumage tracts which we consider here may reside in genes or in specific collocations of cells composing the mechanism of the germ. It seems probable that we are dealing with what may be called "mechanisms" rather than specific quantitative variables in individual genes. These individualized entities or mechanisms comprise the feather germ and all of its potentialities.

In attempting to apply the conception of residual fields to the breast tracts, we must remember that we are dealing with two apparently independent sets of gradient or differential properties at right angles to the anteroposterior axis of the tract. The data show clearly that two variables across the transverse sequence of the breast tracts are not commensurable, i.e., we cannot reduce the data to functions of a single variable expressing changes in the entire transverse sequence.

Our interpretation of the results presented in this paper is then to consider the "incongruous" properties of the transverse sequences to be residual functions of at least two discrete field energies. We may denote each of the two transverse gradient orders (describing continuous and discontinuous properties) as discrete gradient or field orders, and the determination of the properties characterizing each of these gradient orders may accordingly be described as a discrete term in the morphogenetic process.

It follows from this interpretation of the data presented in this paper that at least one of the transverse field differentials need not be effective within the tract as a dynamically determinative and persistent gradient (cf. Huxley and De Beer, 1934). An alternative to this view is that the two differential field functions of the breast tracts represent qualitatively *specific energies* which are continuously (or persistently) effective in the determination of the properties exhibited by the individual regenerating follicle. The possibility that

dynamic differentials of this character actually determine the properties of the individual follicle is at least made highly improbable by the transplantation experiments of Danforth and Foster (1929), although Danforth's (1935) mosaic patterns are possibly due to "persistent" field energies. If, however, one of the determinative differentials of the breast tracts is supposed to be the expression of a fixed, previously determined property of the individual mechanism, there is no good reason why we may not apply similar considerations to all properties exhibited by the follicles of the tract unless we have direct evidence to the contrary. This is only to say that we suppose all properties of the follicles of the breast tract to be embryologically determined and that the residual gradient functions represented in the regenerative properties of individual follicles are evidence for dynamic field orders in the morphogenetic process.

The theoretical significance of the interpretation of fields which we have applied to the plumage tracts can be summarized briefly. We suppose, in the first place, that one definite function of the morphogenetic field is to define discrete properties of individual cells, or of individual mechanisms, e.g., feather follicles. We assume, in the second place, that more than one set of field differentials (energy fields) may operate upon the same individual cell, or collocation of cells, and determine subsequent orders of field residuals which may or may not coincide with spatial co-ordinates of previous residuals.

In the instance of feather regeneration in the breast tracts, the residual properties are evident as quantitative differentials in regenerative capacities or intensities with respect to the mid-ventral axis of the bird's body or the axis of symmetry within the tract.

This concept of the relation between the dynamic field and its residuals[6] simplifies some, at least, of the difficult problems posed by

[6] It is at least theoretically possible that the "individuation" field of Waddington and Schmidt (1933) represents a purely dynamic field, dependent upon and evident as energy relations. The chemical "organizer," on the contrary, is clearly a residual in the sense that we have defined that term. But there is a definite distribution of the organizer (speaking now of the "chemical") within the tissues of the embryo, and in our opinion the order of distribution of the organizer can only be accounted for in the end by reference to rigorously dynamic (or energy) fields. This view has been repeatedly emphasized by Child (see particularly 1924 and 1929) in the same and other connections, but its prime significance has been all too frequently obscured by loosely defined interpretation of gradient and field concepts.

the field concept when the field energy is considered to be a constantly effective (persistent) energy. On theoretical grounds it does not seem necessary to postulate so complex a relation of differentially effective energies as is seemingly required by this view. The concept of discrete terms in field transformations, thenceforth evident as residuals, is a logical alternative in any event.

In view of the theoretically limited time interval involved in the postulated effective or determinative action of dynamic fields from this point of view, we may refer to such fields as *excitation fields*. An excitation field energy, effective for a short time, but attaining a definite and exact end during that time, is at least a theoretically possible statement of the manner in which genetic differentials may be established. The results given in the first part of this paper suggest that specific genetic compositions may be directly referred to field functions from this point of view.

## IV. Summary

1. Analysis of the distribution of properties of individual follicles composing transverse series of the breast tracts (i.e., mediolateral series) gives evidence for the existence of two (independent) gradient orders.

2. These results are discussed as evidences for residual field or gradient functions, and the theoretical relation of excitation and residual fields (or gradients) in the morphogenetic process is briefly touched on.

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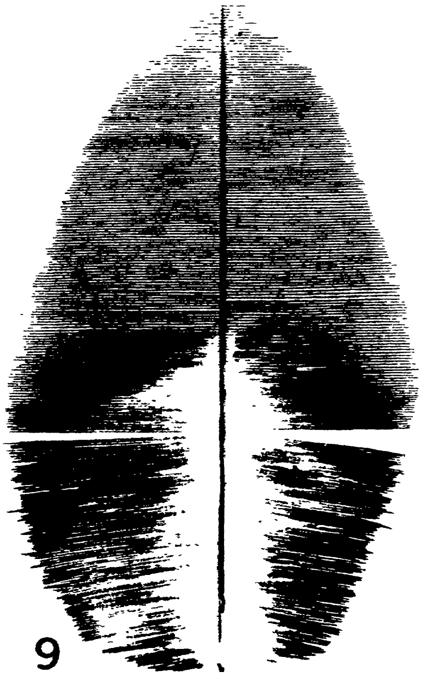
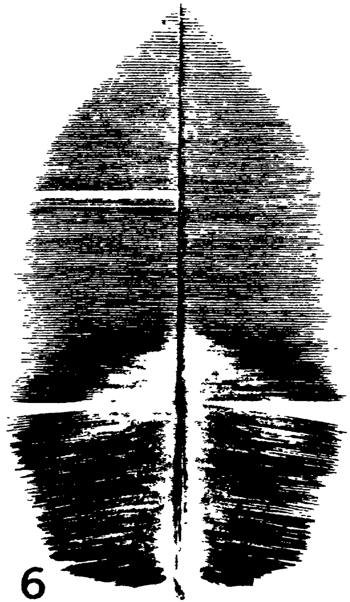
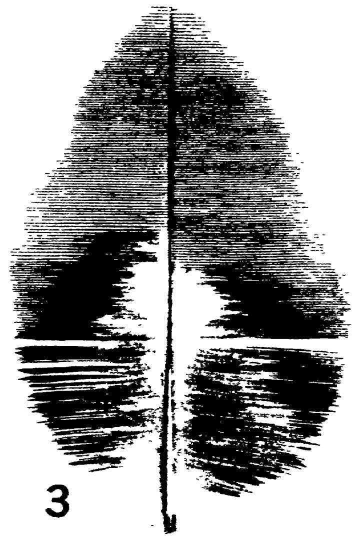
PLATE I



9



6



3

## PLATE I

Photographs of feathers from the third, sixth, and ninth positions of a transverse sequence, left breast tract, Brown Leghorn capon. Feather 6 comes from the approximate axis of symmetry of the tract. Feathers 3 and 9 are each three positions removed from the axis of symmetry. The increasing length of the series (1–9 in mediolateral order) is evident in the lengths of the three feathers reproduced here. Notice also the reversal of symmetry relations in apical contours, in lengths of opposed barbs, and in the asymmetrical distribution of fluff in feathers 3 and 9. In all these characteristics, feather 6 is practically symmetrical.

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## IS FOOD THE EFFECTIVE GROWTH-PROMOTING FACTOR IN HOMOTYPICALLY CONDITIONED WATER?[1]

(One plate)


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THIS is a progress report concerning the results obtained to date in a continuing series of experiments upon mass physiology as a factor in the growth of fishes. The publication of this analysis at this time is justified by the fact that we have arrived at definite conclusions with regard to certain fundamental aspects of the problems involved and by the mutually regretted withdrawal of one of the junior authors (Dr. R. B. Oesting) from active participation in the investigation.

Earlier studies have shown that, other things being equal, and under the conditions of our experiments, isolated goldfishes grow more rapidly if placed in water in which other goldfishes have lived than if in uncontaminated but otherwise similar water. In connection with tests in distilled water, a synthetic pond water was developed in which the total electrolytes were not changed by exposure to fishes for 22 hours under the usual conditions of assay. This synthetic pond water contained 100 mg. $CaCl_2$ and 50 mg. each of $NaNO_3$, $MgSO_4$, and $K_2SO_4$ per liter of high-grade distilled water


[1] We are indebted to Grassyforks Fisheries, Inc., Martinsville, Indiana, for the gift of the goldfishes used in these experiments; and to Miss Gertrude Evans and Mr. Wayne Livengood for access to their unpublished studies on the growth of fishes. This generous co-operation is thoroughly appreciated.

(Allee, Bowen, Welty, and Oesting, 1934). This same formula for synthetic pond water has been used in the present experiments; tests of other waters are in progress.

In general and except as stated, the experimental procedures described in the paper cited above have been followed in the experiments now to be reported, except that in later work glazed earthenware jars holding approximately 4 liters have been substituted for glass aquaria. Throughout the work the commonest variety of single-tailed goldfishes, *Carassius auratus*, has been used exclusively. In recent experiments all have been supplied by the same hatchery.

Including results hitherto published, we have to date a series of twenty-seven paired experiments in which the growth of fishes isolated into untreated conditioned water is compared with that of fishes similarly isolated into comparable, though biologically uncontaminated, control water. In all we have records (through H7) from 290 experimental and 274 control fishes that lived through the 20–30 days of assay for this experiment alone. With all of these, the full conditioned water, not sieved or filtered, was used to test its effect on growth; faeces, slime, and regurgitated food particles were all present indiscriminately as they were introduced by the conditioning fishes. It is worth noting specifically that the conditioning fishes were fed daily in water other than that which they were conditioning. After 2 hours of feeding they were washed until traces of food were removed from their bodies and until the first regurgitations, if any, were also removed, and were finally rinsed briefly in distilled water before being again used as conditioning fishes for the following day.

Growth was determined by the photographic method described in the report cited above. Photographs were taken under standard conditions at the beginning and at the end of the assay period. These were measured to determine growth in length from anterior margin of the eye to the base of the tail, and also at the region of greatest width. All photographs are on file. Since the photographs are slightly magnified, the growth as recorded is to be regarded as in artificial units which must be multiplied by 0.82 to obtain the actual growth of the fishes in millimeters. Critical measurements in the present report are in length and have an accuracy of $\pm 0.04$ per cent,

which makes the actual error for the fishes used approximately $\pm 0.02$ units.

For the growth experiments with full-conditioned and otherwise untreated conditioned water, there was a mean growth of 1.8 units for the experimental fishes and of $-0.23$ units for the accompanying controls. Each of the twenty-seven available experiments has the mean difference in the same direction, and in all but three of the individual experiments the results are statistically significant.[2] Taken as a whole, the mean difference in growth of 2.03 units has $P = < 0.0001$, which means that there is less than one chance in ten thousand of obtaining similar results by random sampling.

As stated in the previous report, in an attempt to eliminate grosser particles of faeces and regurgitated food, the conditioned water was strained through a phospho-bronze screen with 325 meshes per inch; the openings average 0.040 mm. The control water was similarly strained through another and wholly similar sieve. We now have a total of fifteen paired experiments involving in all 180 fishes assayed in the sieved conditioned water and 142 accompanying controls. The former grew 1.85 units in length; the latter, 1.00 unit. The mean difference of 0.85 has a $P$-value of $< 0.0001$ and is statistically significant.

These two sets of experiments have been made with the fishes in conditioned water, all of which had been exposed to the conditioning fishes. We have continued to gain experience with the use of a smaller amount of the conditioning water, which became much more highly charged with organic materials obtained from the contained fishes. A certain amount of this water, usually 190 cc. per 2 liters, was added to water similar to that in the controls to make the conditioned water used in the growth assays. We now have data from fifteen such experiments with 210 experimental and 130 control fishes. The fishes in the conditioned water grew 2.28 units; those from the accompanying controls grew 1.28 units. The difference of 1.00 has a $P$ value of $< 0.0001$.

These three types of experiments extend and confirm the findings

[2] In this paper statistical significance is given in terms of $P$ and is based on "Student's" method for finding statistical significance of relatively small numbers of paired observations. $P = 0.05$ is equivalent to three times the probable error; this value is taken as the extreme upper limit of statistical significance.

already reported. The additional data on these points have been accumulated incidentally as normal or conditioned water controls while prosecuting analysis of the nature of the conditioning factor, some of the results of which will be presented in the following pages; others will be reported elsewhere. There is no doubt but that under the conditions of our experiments goldfishes of about an inch in body length grow faster in 20–30 days of assay when in freshly prepared and daily changed homotypically conditioned water than when in similar but biologically uncontaminated water (*a*) when nothing is removed from the water, (*b*) when particles larger than 40 $\mu$ are screened out, and (*c*) when a small amount of water is heavily conditioned and screened and then diluted to the usual conditioning strength.

We have some evidence which indicates that there is less difference in growth of fishes in homotypically conditioned water, as compared with that of accompanying control fishes, when the latter show extra-good or superb growth during the assay period. In part, at least, this seems to be a matter of the amount and kind of food given the assay fishes. Further and more exact discussion of this problem is reserved for the present.

*Effects of different amounts of conditioning.*—In the general work on the effect of biological conditioning of the water on various biological processes and in early work on the present problem, it was found to be comparatively easy to demonstrate that overconditioning produces harmful effects (Allee, 1931, 1934); in terms of the present experiments, such overconditioning retards, rather than promotes, growth. The retarding effect is usually attributed to the effect of the presence of metabolic wastes or to decomposition products of these. This immediately raises the question concerning whether the amount of conditioning used in these experiments was above or below the optimal amount.

The amount of conditioning used here was in all cases that which could be accomplished in about 22 hours. No food was added directly to the water being conditioned, since the conditioning fishes were fed in other water during the two remaining hours of the twenty-four. The amount of conditioning is approximately a factor of the number and the size of fishes present, together with the volume

of the water to which they were exposed, and may be indicated by a "conditioning coefficient." This coefficient is obtained by dividing the product of the number of conditioning fishes times their length in millimeters by the number of liters of water conditioned. In the experiments summarized in the preceding pages this coefficient varied from 12 to 74; 45 of the 58 lots of water had a conditioning coefficient of between 20 and 30; 6 were over 40; and the highest three were 49, 52, and 74, respectively. For example, in Experiment 7 there were approximately 0.165 fishes 76 mm. long per liter of water; and in Experiment 19 there were 0.95 fishes 47 mm. long per liter for the same amount of water. The former gives a conditioning coefficient of 12.5; the latter, one of 44.7.

When the amount of growth in any given experiment is plotted against the conditioning coefficient, it was found that in these experiments 24 is about the median value. Such a plotting reveals a wide variation in growth-rate at any given level of conditioning, as might be expected considering the number of known variables. The mean growth in the twenty-three experiments below the median conditioning coefficient was 1.57 units; for the eight experiments at the median, the mean growth was 2.11 units; for the twenty-six experiments above this coefficient, it was 2.17. (The exact conditioning coefficient of one experiment is not now known.) These results indicate that we have not been overconditioning the medium, at least in the majority of the experiments; instead there has been too little, rather than too much, conditioning for most rapid growth.

One experiment (H-7) was devised in part to test the effect of different amounts of conditioning upon the rate of growth. The results obtained are summarized in Table I. The same concentrated conditioned water was used in all, but the proportions added to make up the assay volume of 2 liters was varied to give the conditioning coefficients of 25, 49, and 74, respectively. Under these conditions there was the most rapid growth at the intermediate strength used, which was significantly greater than the growth given with the most strongly conditioned water.

This direct test supports the conclusions reached from a study of the growth in relation to the amount of conditioning in the other ex-

periments; certainly, in general, we have been working below, rather than over, the conditioning value which gives the most rapid growth under the conditions of these experiments. The exact optimum amount of conditioning has not been established.

The fed conditioning fishes were given a varied and adequate diet, which, with one exception, contained either *Daphnia* or fresh liver or both. Although the effect of the diet fed the conditioning fishes deserves direct experimental analysis, this has not been made as yet, beyond the tests to be reported later in the present paper, in which the conditioning fishes were starved. When the water was conditioned by starved fishes, the animals to be used in a given experiment were starved for 5 days before the experiment was begun,

TABLE I

THE EFFECT OF DIFFERENT AMOUNTS OF CONDITIONING UPON RATE OF GROWTH IN GOLDFISHES IN AN ASSAY PERIOD OF 20 DAYS

| Columns | Observed Growth | | | Statistical Probabilities | | |
|---|---|---|---|---|---|---|
| | i | ii | iii | ii−i | ii−iii | i−iii |
| Conditioning coefficients... | 25 | 49 | 74 | ........ | ........ | ........ |
| Growth in length......... | 1.5 | 1.9 | 1.2 | 0.19 | 0.03 | 0.32 |
| Growth in length plus width | 1.7 | 2.4 | 1.4 | 0.13 | 0.045 | 0.48 |

and were used as conditioning fishes for a 10-day period, when a similarly prepared lot were substituted. In the first of these tests, the same lot of fasting fishes was used throughout.

There are several lines of evidence that food materials are present in the conditioned water whether screened or unscreened. In the first place, such water is opalescent and contains strands of mucus and other suspended particles visible to the eye. The appearance of the water changes when the conditioning fishes are fed liver, as compared with rolled oats. Faeces are present in the unscreened water in relatively large pieces. These are ingested at times, as shown by the following observations: Two fishes, starved for 4 days, were placed in 500 cc. of synthetic pond water which contained four pieces of faeces on which flecks of charcoal had been carefully placed. The next morning there was but one piece of faeces in the container, and

this one had charcoal on the inside of the coil and was coated on the outside with the usual jelly-like characteristic of goldfish faeces.

Two other fishes starved for 4 days were placed in the same amount of synthetic pond water which contained suspended charcoal that had passed through one of the 325-mesh phospho-bronze sieves in regular use for straining this water. The faeces of these fishes contained charcoal the following morning; this shows that particles may be ingested even when they are 40 $\mu$ or less in diameter. This experiment has been repeated twice, once with identical results and again when the fishes yielded either no faeces or clear jelly-like strands only.

Attempts to assay the food value of conditioned water include (*a*) measurement of required oxygen; (*b*) spectrographic analysis; (*c*) conditioning by fasting fishes; (*d*) filtering the conditioned water; (*e*) comparison with results obtained by the addition of minute particles of food in direct conditioning, and (*f*) assays to determine possible vitamin effects from the fish-conditioned water. Results from (*e*) and (*f*) will be reported elsewhere. It is pertinent here to inquire whether the observed increase in growth of fishes in conditioned water is caused wholly or in part by increased food intake.

*Required oxygen.*—Required oxygen measures the total amount of oxygen necessary to oxidize the organic material present to a fairly stable end-product. This gives a measure of the total amount of oxidizable organic matter present, but does not, of course, measure its availability as food. The method used was that described in *Official and Tentative Methods of Analysis*, of the Association of Official Agricultural Chemists (1925), p. 87. Results are given in milligrams of oxygen consumed per liter when the period of boiling with permanganate is limited to 10 minutes. Tests were usually made at ten times the assay strength; these give results similar to those in H-4, in which the tests were made on the assay-strength water. In the latter experiment the observed differences were too slight to be measured with certainty by this method, but in each case they were in the same direction as were the similar differences obtained in the other experiments. The data for the required oxygen and for growth in the several types of water tested are summarized in Table II.

STATISTICAL PROBABILITIES

| | |
|---|---|
| V–IX, $P=0.0016$ | IV–XII, $P=0\ 9$ |
| VI–X, $P=0.0094$ | VIII–XII, $P=1.0-$ |
| VI–XII, $P=<0.0001$ | IV–X, $P=0.558$ |
| X–XII, $P=0.0134$ | |

Study of this table shows that the controls growing in water which at no time absorbed oxygen grew, on the average, 1.55 units. Significantly increased rates of growth above this level must be correlated with some aspect of the conditioning effect. The differences in growth in filtered water, whether from fed or from starved conditioning fishes, is not significant; but there are too few cases recorded in this table for this to be taken as a final result. The fishes in water from starved and from fed conditioning fishes which had been sieved, rather than filtered, grew significantly more than did those in the

TABLE II

REQUIRED OXYGEN IN FISH-CONDITIONED WATER IN RELATION TO FISH GROWTH IN SUCH WATER GIVEN IN TERMS OF STRENGTH CALCULATED FOR THE WATERS TO WHICH THE FISHES WERE EXPOSED

| Experiment No. | Conditioning Coefficient | Conditioning Fishes—Fed | | | | Conditioning Fishes—Starved | | | | Control | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Filtered | | Sieved | | Filtered | | Sieved | | | |
| | | Required Oxygen (Mg.) | Growth in Length (Mm.) | Required Oxygen (Mg.) | Growth in Length (Mm.) | Required Oxygen (Mg.) | Growth in Length (Mm.) | Required Oxygen (Mg.) | Growth in Length (Mm.) | Required Oxygen (Mg.) | Growth in Length (Mm.) |
| i | ii | iii | iv | v | vi | vii | viii | ix | x | xi | xii |
| H-1... | 13 | ...... | ...... | 0.16 | 4.22 | ...... | ...... | 0.08 | 3.86 | 0 0 | 3.6 |
| H-2... | 24 | 0.28 | 3.70 | 0.35 | 5.10 | 0.08 | 4.00 | 0.08 | 4 80 | 0.0 | 4.0 |
| H-3.. | 26 | 0.43 | 1.66 | 0.51 | 1.44 | 0.13 | 1.54 | 0.13 | 1.53 | 0.0 | 1.1 |
| H-3... | 26 | 0.43 | 1.14 | 0.51 | 1.52 | 0 13 | 1.23 | 0.13 | 1.84 | 0.0 | 1.1 |
| H-4... | 26 | 0.44 | 4.27 | 0.65 | 4 76 | 0.09 | 3.30 | 0.16 | 3.80 | 0.0 | 4.2 |
| H-5... | 27 | 0.72 | 2 10 | 0.80 | 2.10 | 0.15 | 1 40 | 0 18 | 1.20 | 0.0 | 1.1 |
| H-6... | 27 | 0 64 | 1.00 | 0.74 | 2.00 | ...... | ...... | 0.25 | 0.90 | 0.0 | 0 7 |
| H-6... | 26 | ...... | ...... | 1.16 | 1.40 | ...... | ...... | ...... | ...... | 0 0 | 0.8 |
| H-6... | 52 | ...... | ...... | 2 32 | 1 60 | ...... | ...... | 0.50 | 0.90 | 0.0 | 0.96 |
| H-7... | 25 | ...... | ...... | 0.62 | 1.50 | ...... | ...... | 0.15 | 0.60 | 0.0 | 0.3 |
| H-7... | 49 | ...... | ...... | 1.24 | 1.90 | ...... | ...... | 0.30 | 0.70 | 0.0 | 0.7 |
| H-7... | 74 | ...... | ...... | 1.88 | 1.20 | ...... | ...... | 0.45 | 1.00 | 0.0 | 0.0 |
| Mean | ...... | 0 49 | 2 31 | 0.91 | 2.40 | 0.12 | 2.29 | 0.22 | 1.92 | 0.0 | 1.55 |

controls; and those in water conditioned by fed fishes grew more than those from the water conditioned by starved fishes. The recorded differences for these fishes are statistically significant both as regards growth and differences in required oxygen; the greater the mean amount of required oxygen, the more growth. Stating this in other terms, the greater the possible food value of the conditioned water, the greater the growth, except when filtered conditioned water was tested; and in the few tests recorded here these filtered waters did not promote growth significantly better than did the control water, even though one set required double the amount of oxygen, as did the sieved water from starved conditioned fishes, which did stimulate growth significantly.

In order to test the matter further, the correlation coefficient was calculated for the amount of increase in growth of the experimental fishes in sieved conditioned water, whether from fed or from starved fishes, over that in the accompanying controls. There is a slight, but apparently trustworthy, positive correlation, with $r = 0.066 \pm$ a standard error of 0.021.

*Conditioning by fasting fishes.*—Another method of testing whether the beneficial effects of conditioned water of the strength used resulted from the addition of food values by the conditioning fishes is to starve the fishes used to condition the water. As already stated, such conditioning fishes were starved for 5 days before the experiment began, and after the first trial were replaced at the end of each 10-day period by other fishes that had also been starved for a preliminary period of 5 days. This change was made because of the possibility that the fasting fishes might well cease producing as much slime, or other conditioning factors other than food, as would normally fed fishes. This water conditioned by fasting fishes was screened as usual, and the experiments were conducted in all details according to standard practise. The conditioning coefficient was 27 or less in ten of the twelve comparable experiments and went above 70 in the most highly conditioned one.

It is probably important in this connection that, while with fed conditioning fishes this highly conditioned water was definitely more harmful than was water with a conditioning coefficient of about 50, with these starved fishes the growth was decidedly better than with

the lower concentrations and was, in fact, the only experiment of this series in which there was statistically significant growth in any one experiment considered alone.

The data for these experiments are summarized in Table II, which was presented in another connection in an earlier section. This table gives the results from 161 experimental and 114 control fishes. Despite the lack of statistical significance in any individual experiment except when the most highly conditioned water was used, when all are considered as twelve paired experiments the difference in growth of 0.37 units (columns x -xii in Table II) has a statistical probability of 0.0134, which must be taken seriously.

If the food introduced by conditioning fishes is the principal factor in promoting growth in homotypically conditioned water, then, other conditions being equal, water in which fasting fishes have lived should have less growth-promoting power than if it is conditioned by fed fishes. The experiments summarized in columns vi and x of Table II give the available data on this point; in all of these, comparable fishes were assayed simultaneously in the two types of conditioned waters. Because of differences in growth-rates at different times and because of possible variations in environmental conditions in the laboratory, it is not safe to rely for critical results on growth differences between groups tested at different times. For the slight changes with which we are dealing, paired experiments are much safer.

With the eleven paired experiments at hand, there was greater mean growth of 0.48 units in the water conditioned by fed fishes. The statistical probability is 0.0094. In these experiments, placing assay fishes in water conditioned by fasting fishes increased their growth-rate during the assay period slightly but significantly. The use of water conditioned by fed fishes caused a somewhat greater and somewhat more significant increase in growth over and above that produced by the water from fasting fishes.

It is significant for our problem to note that an increase of required oxygen from 0.0 to 0.22 cc. per liter over the untreated controls produced almost as great a mean increase in growth as did an increase from 0.22 to 0.91 cc. of required oxygen per liter. Either the conditioning fishes introduce some growth-promoting substance other

than food into the water or the amount of growth is relatively much more stimulated by the presence of a very slight initial amount of food material than it is by further and more marked increases. This problem must be carried forward into the next section.

*Filtered conditioned water.*—Another method of removing the possible food value of the conditioned water is by filtering. The effect of this treatment on growth is summarized in Table III, which records

TABLE III

SHOWING THE EFFECT OF FILTERING OF CONDITIONED WATER ON GROWTH; IN O THROUGH ONE NO. 40 WHATMAN PAPER, IN H THROUGH TWO S. AND S. NO. 595 PAPERS

| Experiment No. | Days Tested | Conditioned by Fasting Fishes | | Control | | *P* | Conditioning Coefficient | Conditioned by Fed Fishes | |
|---|---|---|---|---|---|---|---|---|---|
| | | No. of Fishes | Growth (Mm.) | Growth (Mm.) | No. of Fishes | | | No. of Fishes | Growth (Mm.) |
| O-11E | 30 | 10 | 4.5 | 3.1 | 9 | None | 24 | ...... | ...... |
| O-11D | 30 | 10 | 5.9 | 3.7 | 10 | 0 007 | 24 | 9 | 3.1 |
| O-11k | 30 | 10 | 4.7 | 3.6 | 10 | None | 24 | ...... | ...... |
| O-11m | 30 | 10 | 4.4 | 3.3 | 10 | None | 24 | ...... | ...... |
| O-12AB | 20 | 7 | 0.06 | −0.03 | 7 | None | 17 | ...... | ...... |
| O-12eD | 20 | 7 | 0.0 | 0.7 | 5 | None | 17 | ...... | ...... |
| O-12Lk | 20 | 10 | 0.7 | 0.6 | 5 | None | 17 | ...... | ...... |
| O-12nm | 20 | 5 | 0.2 | 0.3 | 8 | None | 17 | ...... | ...... |
| H-2 | 30 | 15 | 4.0 | 3.9 | 15 | None | 24 | 15 | 3.70 |
| H-3 | 20 | 9 | 1.54 | 1.1 | 10 | None | 26 | 10 | 1.66 |
| H-3 | 20 | 9 | 1.23 | 1.1 | 10 | None | 26 | 10 | 1.14 |
| H-4 | 20 | 9 | 3.3 | 4.2 | 9 | None | 26 | 10 | 4.27 |
| H-5 | 20 | 15 | 1.4 | 1.1 | 15 | None | 26 | 15 | 2.10 |
| Total (13) | 310 | 126 | 2.46 | 2.0 | 123 | 0.0816 | ..... | 69 | 2.66 |

the results from thirteen experiments with filtered conditioned water from fasting fishes and of six experiments with similarly filtered water from fed conditioning fishes which were made simultaneously with some of the experiments on water from fasting fishes. In the experiments marked "O" the water was filtered through one thickness of Whatman No. 40 filter paper; in those marked "H," through two thicknesses of No. 595 Schleicher and Schull paper.

Required oxygen was not determined for all these experiments. Turning again to Table II, one finds that, for those determinations

made, the filtered water from fasting fishes required 0.12 mg. oxygen per liter and the filtered water from fed conditioning fishes required approximately four times as much. Neither grew significantly more than did the controls, and in the paired experiments the two lots grew almost exactly the same whether in one or the other filtered conditioned water. When all the experiments from filtered water from fasting conditioning fishes are considered, the data summarized in Table III show that the observed increase in growth in the conditioned water of 0.46 units approaches, but by no means reaches, statistical significance.

Other experiments are available on the effect of filtering water from fed conditioning fishes which were not made at the same time as those summarized in Table III. When all these are considered with their respective controls as one series of twenty-one paired experiments, there is a mean increase in growth of 0.36 units, with $P=0.007$ and hence significant statistically.

The results obtained with these filtered conditioned waters can be explained if one assumes either that the filtering removes some growth-promoting substance or that it takes out the food particles which are of sufficient size to be of value as fish food although it does not remove all the organic matter. According to the required-oxygen data (Table II), filtering removes about half of the organic material from the water, whether it comes from fed or from fasting conditioning fishes. We are not yet in a position to decide with certainty between these two possibilities, which will be discussed again later in the present report.

*Protein extract.*—If food is the primary growth-promoting factor in conditioned water, exceedingly small amounts of extract from the skin of goldfishes would not be expected to have an effect in stimulating growth. On the other hand, if there is some sort of growth stimulant, other than food, in the homotypically conditioned water, one of the possible sources would be found in the secretions given off from the surface of the fishes. This offers a new and attractive avenue of approach to the whole problem, which has been and is being investigated.

A protein extract of the surface of goldfishes was prepared as follows: Fifteen goldfishes, averaging 50 mm. from tip to origin of tail

fin, were placed in an acetate buffer at pH 4.15–4.20. At this pH the slime coating on the bodies was precipitated as a white material, which was scraped off, washed, and then dissolved in 10 cc. of N/2 NaOH plus 25 cc. distilled water. The solution was filtered and adjusted to pH 4.2 with N/2 $H_2SO_4$. The resulting protein precipitate was washed by decantation and dissolved in 10 cc. of N/2 NaOH; enough N/2 $H_2SO_4$ was added to neutralize the solution. This solution was diluted to 100 cc. and autoclaved for 10 minutes at 15 pounds pressure. The prepared solution was kept at 6° C., and 1 cc. per liter per day was added to the usual synthetic pond water for one set of assay fishes.

The solution gave a strong protein test and had the odor of fish (amines). As an added control, 10 cc. of N/2 NaOH plus 10 cc. of N/2 $H_2SO_4$ was diluted to 100 cc. with distilled water, autoclaved with the protein solution, and kept in the icebox. Again 1 cc. per liter per day of this salt control was added to the medium of the lot of isolated fishes that made up the so-called "salt-control."

A material with protein properties was isolated in very small amounts from the usual fish-conditioned water. The protein extract and this material from fish-conditioned water showed the reactions summarized in Table IV.

The protein solution so prepared was used in Experiment O-1. In Experiment O-2, preparation was similar except that 66 fishes averaging 65 mm. in length were extracted. The final solution, after neutralization, was diluted to 180 cc., autoclaved, and stored as before. For Experiment O-4, 20 large goldfishes, 80 mm. long, were extracted. After the first filtration the precipitate was redissolved, re-washed, and reprecipitated two different times, made up to 150 cc., and autoclaved. 0.5 cc. was added per liter, and a 2-liter assay volume was used.

In Experiment O-5, 166 fishes 66 mm. long were extracted as before, except that the second precipitate was thoroughly washed in distilled water, then in alcohol, followed by ether; then desiccated *in vacuo* for 5 days. Of this protein, 1.040 gm. were dissolved in 25.0 cc. of N/10 NaOH plus 175 cc. of distilled water, making a solution of approximately 0.5 per cent, 1 cc. of which was added to 2 liters of water to make a dilution of 1:400,000. The same method was fol-

lowed in later experiments; only the dilutions were varied. Other modified controls were also applied to provide adequate chemical controls.

The summary of results obtained in growth assays is given in Table V. Section A of this table contrasts growth in water plus the protein extract with growth in the usual control; B presents the contrast between the dilute protein extract and the various salt controls; C shows the one test made to date on the relative efficiency of two dilutions of the protein extract; and D compares growth in the

TABLE IV

COMPARABLE TESTS ON PROTEIN FROM AN EXTRACT FROM THE SURFACE OF FISHES AND FROM ORDINARY FISH-CONDITIONED WATER

| Tests | Protein Extract | Conditioned Water |
|---|---|---|
| Xanthoproteic | Positive | Positive |
| Biuret | Positive | Positive |
| Molisch | Positive | Not tested |
| Millon | Positive | Positive |
| Hopkins-Cole | Faint positive | Not tested |
| Lead acetate | Negative | Negative |
| Glyoxalic | Not tested | Positive |
| Color, solution | Pale brown | ..................... |
| Color, solid | White | ..................... |
| Remarks | On drying in air, obtained brown odorous resin-like material difficult to dissolve even in strong alkali with heat | Not coagulated by heat; insoluble in acetic acid, with heating in strong HCl; dissolved in alkaline solution |

diluted protein extract with that in the usual strength of conditioned water.

In the experiments available to date there is the following pertinent evidence: (*a*) The difference in growth between assay fishes in water plus protein extract and growth in untreated but otherwise similar control water is 1.41 units, with $P=0.0106$. (*b*) The difference between the growth of fishes in the dilute protein extract and the growth of fishes in all salt controls is 2.61 units, with $P=<0.0006$. (*c*) In the one experiment available there is no significant difference between the growth in 1:400,000 and 1:800,000 parts of protein in the assay water. (If other results should turn out to be

## TABLE V

### EFFECT OF PROTEIN EXTRACT FROM SKIN OF GOLDFISHES ON GROWTH OF GOLDFISHES

| Experiment No. | Days Treated | Extract Conditioned: No. of Fishes | Extract Conditioned: Growth | Control Otherwise Unconditioned: Growth | Control Otherwise Unconditioned: No. of Fishes | *P* | Strength of Extract | Salts |
|---|---|---|---|---|---|---|---|---|
| **A. Protein Extract versus Ordinary Control** | | | | | | | | |
| O-3 | 10 | 3 | 1.87 | 0.33 | 3 | 0.068 | ? | ........ |
| O-4 | 20 | 6 | 2.16 | 0.43 | 9 | ........ | ? | ........ |
| O-6 | 20 | 8 | 0.62 | 0.05 | 8 | 0.0442 | 1:800000 | ........ |
| O-7 | 30 | 9 | 1.26 | −0.10 | 10 | ........ | 1:400000 | ........ |
| O-7 | 30 | 10 | 0.76 | −0.10 | 10 | ........ | 1:800000 | ........ |
| O-8 | 30 | 10 | 3.03 | −0.05 | 9 | 0.0004 | 1:400000 | ........ |
| O-9 | 30 | 10 | 3.93 | 3.23 | 10 | 0.30 | 1:700000 | ........ |
| Totals or means | ...... | 56 | 1.95 | 0.54 | 59 | *P*=0.0106 | ........ | ........ |
| **B. Protein Extract versus Salt Control** | | | | | | | | |
| O-1 | 20 | 3 | 7.1 | 0.13 | 3 | 0.0074 | ........ | ........ |
| O-2 | 20 | 5 | 3.68 | 0.64 | 5 | 0.0028 | ........ | ........ |
| O-4 | 20 | 6 | 2.16 | 0.10 | 6 | 0.0030 | ........ | ........ |
| O-5 | 20 | 8 | 3.8 | 0.9 | 8 | 0.0036 | ........ | NaCl |
| | | | | 0.7 | 8 | 0.0016 | 1:400000 | Amines |
| | | | | 0.4 | 8 | 0.0003 | 1:400000 | $CaCl_2$ |
| | | | | 0.6 | 8 | 0.0003 | 1:400000 | $NaNO_2+(NH_4)_2SO_4$ |
| O-7 | 30 | 9 | 1.26 | −0.05 | 10 | 0.0040 | 1:400000 | $CaCl_2$ |
| | | | | −0.02 | 10 | 0.0040 | 1:400000 | $NO_2+NH_4$ |
| ........ | 30 | 10 | 0.76 | −0.05 | 10 | 0.0012 | 1:800000 | $CaCl_2$ |
| | | | | −0.02 | 10 | 0.0108 | 1:800000 | $NO_2+NH_4$ |
| O-8 | 30 | 10 | 3.03 | 0.25 | 9 | 0.006 | 1:400000 | $NaCl_2$ |
| | | | | 1.15 | 9 | 0.0316 | 1:400000 | $NaNO_2+(NH_4)_2SO_4$ |
| | | | | 0.40 | 8 | 0.0052 | 1:400000 | $Na_2SO_4$ |
| O-9 | 30 | 10 | 3.93 | 4.09 | 10 | Not sign. | 1:700000 | Salt control |
| Totals or means | 200 | 61 | 3.22 | 0.61 | 122 | 0.0006 | ........ | ........ |
| **C. 1:400000 versus 1:800000 Protein Extract** | | | | | | | | |
| O-7 | 30 | 9 | 1.26 | 0.76 | 10 | 0.27 | ........ | ........ |

TABLE V—*Continued*

| Experiment No. | Days Treated | Extract Conditioned | | Control Otherwise Unconditioned | | P | Strength of Extract | Salts |
|---|---|---|---|---|---|---|---|---|
| | | No. of Fishes | Growth | Growth | No. of Fishes | | | |
| D. Protein Extract versus Conditioned Water | | | | | | | | |
| O-3.... | 10 | 3 | 1.87 | 1.3 | 3 | 0.046 | ? | .................. |
| O-4. .. | 20 | 6 | 2 16 | 1 08 | 8 | None | ? | ... .............. |
| O-6.... | 20 | 8 | 0.62 | 0.66 | 7 | None | 1:800000 | .................. |
| O-8..... | 30 | 9 | 3.03 | 3.23 | 10 | None | 1:400000 | .................. |
| Totals or means | ...... | 26 | 1.92 | 1.55 | 28 | $P=0.26$ | ...... .. | .................. |

consistently in the same direction, the observed difference of 0.5 units more growth in the stronger solution would become significant; this, however, cannot be assumed until tested.) Finally, (*d*) the observed difference in growth in the protein-conditioned water and that in ordinary fish-conditioned water is not significant.

With regard to the food value of the protein-conditioned water we have little evidence. Two tests of the required oxygen were made in Experiment O-10 which showed an oxygen requirement of 1.36 mg. per liter, which is to be compared with a mean oxygen requirement of 0.91 mg. per liter for four accompanying tests in ordinary conditioned water. The strength of the protein extract tested was ten times the assay strength; hence the comparable values with required oxygen, as shown in Table II, would be 0.136, which puts the organic value in the general range of that given in the water conditioned by fasting fishes and then filtered.

If we attempt to explain the stimulation produced by these protein extracts as a result of added food value, we have two major difficulties. The first has already been mentioned in part and concerned very slight growth-promoting power of filtered conditioned water even from fed fishes with approximately four times the required oxygen value of this water. If we assume that the filtered conditioned

water has little power to promote growth because the particles remaining after filtering are too small to be of much food value to the fishes, we have our second major difficulty, since this growth-stimulating protein is probably present as a true solution.

Further, if this is an effect produced by the caloric value of the protein as food, it is extremely potent material, since a dilution of 1:400,000, or even of 1:800,000, has a stimulating effect. It seems more reasonable to assume that the protein acts as some other sort of growth stimulant. We are prepared for the latter assumption by the work of Peebles (1929) on the effect of the protein fraction from a self-conditioned water in stimulating the growth of echinoderm embryos.

This is not the place to give in detail the studies to date on the nature of the protein. In summary these tests show:

Amide $N_2$ makes 1.26 per cent of the sample.
Melanoidin $N_2$=0.90 per cent of the sample.
Diamino $N_2$=1.97 per cent of the sample.
Monoamino $N_2$=10.43 per cent of the sample.
Total $N_2$=14.57 per cent of the sample.
Alpha amino $N_2$=80.55 per cent of the total nitrogen.

One other exploratory test has been made: Ovomucin was prepared according to directions in use in routine work in physiological chemistry at this university. In 17 individual pairs of test fishes there was no significant difference in growth in water plus ovomucin and growth in water plus protein extract; neither was there a significant difference in the same number of cases between fishes in ovomucin and in ordinary control water. The simultaneously made comparisons between fish growth in the protein extract and in untreated control water were statistically significant.

*Charcoal treatment.*—Preliminary investigation of the effect of treating concentrated conditioned water with charcoal and then testing the growth-promoting power of the treated water and of water conditioned by adding material or materials presumably protein in nature, eluded from the charcoal, have shown that this is a promising lead. The data at hand must not be taken too seriously and hence are not given in detail; they do indicate that the charcoal adsorbs something from the fish-conditioned water which can be

## PLATE I

### Absorption Spectra

Fig. 1.—*A*, water conditioned by fishes that had been fasting for 5 days; *B*, untreated control water.

Fig. 2.—*A*, concentrated conditioned water from fishes fed liver; *B*, aqueous extract of liver; *C*, protein extract from surface of goldfishes, purified and dissolved in control water.

Fig. 3.—*A*, concentrated conditioned water from fed fishes; *B*, protein extract from surface of goldfishes, purified and dissolved in control water; *C*, water as in *A* after treatment with activated charcoal and filtering.

Fig. 4.—*A*, concentrated conditioned water from fishes fed rolled oats; *B*, aqueous extract of rolled oats; *C*, untreated control water.

PLATE I

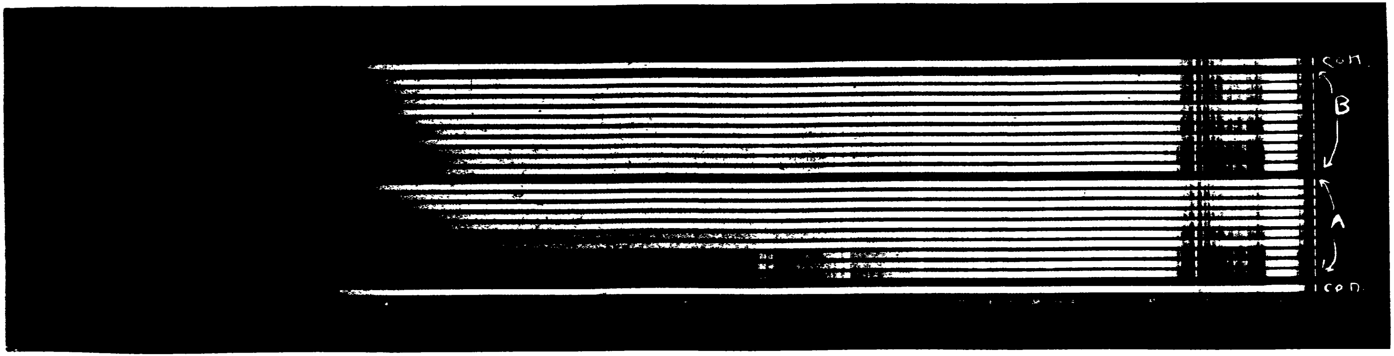
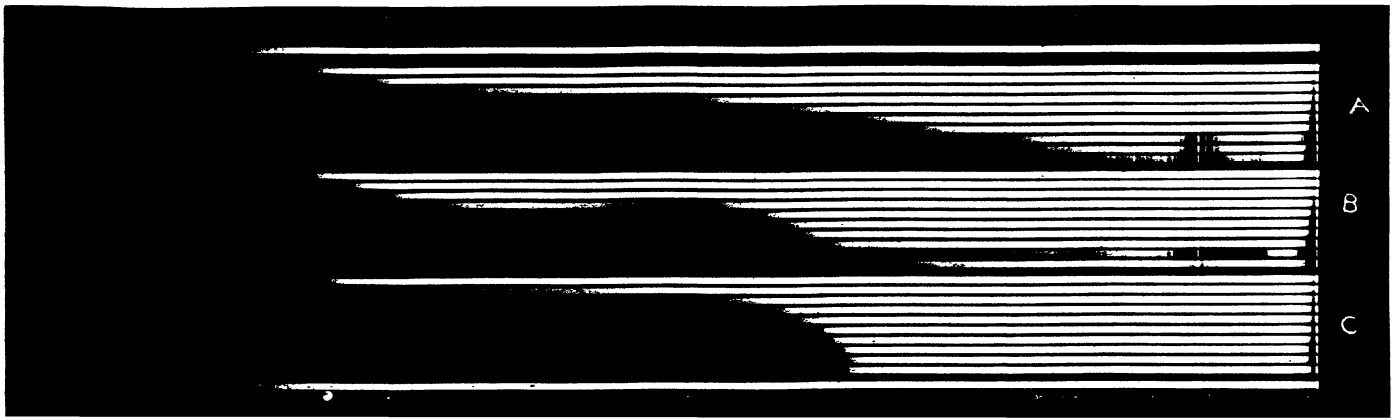



FIG. 1



spectra are different from those of liver-fed fishes, from water extracts of liver, or from protein fractions of mucin from the surfaces of fishes. Oat-fed-fish-conditioned water and aqueous extracts of oats are very much alike.

Comparisons of these spectra with the oxygen absorption of the various solutions or suspensions gives a semiquantitative basis which aids in comparisons. These values are shown in Table VI.

TABLE VI

OXYGEN ABSORPTIONS OF WATERS UPON WHICH ABSORPTION SPECTRA HAVE BEEN REPORTED

The Required Oxygen Values Given Here Are Ten Times Those in Table II, Which Were Shown at Assay Strengths

| Figure No. of Plate I | Spectrum Letter | Oxygen Absorbed per Liter (in Mg.) |
|---|---|---|
| 1.................. | *A* | 2.90 |
| | *B* | 0.00 |
| 2.................. | *A* | 11.37 |
| | *B* | 7.39 |
| | *C* | 19.38 |
| 3.................. | *A* | 11.05 |
| | *B* | 19.38 |
| | *C* | 2.15 |
| 4.................. | *A* | 5.11 |
| | *B* | 4.05 |
| | *C* | 0.00 |

These data demonstrate the presence of organic matter of some type in the water conditioned by fed fishes and show the relative lack of such materials in the water conditioned by fasting fishes (*A* of Fig. 1, Pl. I). The evidence regarding the presence of a significant amount of mucin from the surfaces of the fishes in the usual fish-conditioned water is inconclusive; further work at this point is needed and is in progress at this time. These absorption spectra have been confirmed a sufficient number of times to assure us that they are repeatable and are valuable as aids in interpreting our biological assays.

## DISCUSSION AND SUMMARY

The present report centers about the question taken as the title: Is food the effective growth-promoting factor in homotypically conditioned water? The problem is being attacked in this laboratory along three main lines, two of which will be reported elsewhere and by others. The most direct approach to the problem is furnished by suspending various amounts of different food substances in water as watery suspensions or extracts and then removing different fractions and testing the different effects on growth of such food-conditioned waters. A second approach is directed toward determining whether a food accessory is introduced as a part of the conditioning process.

The evidence we have presented demonstrates the presence of particles of food in the conditioned water whether whole or screened through openings of about 40 $\mu$ in diameter. The nature of the foodstuffs varies with different diets and comes from regurgitated particles and from the faeces. When rolled oats is the chief constituent of the diet, the concentrated conditioned water becomes cloudy with regurgitated oat particles. If the diet is chiefly of liver, the water is more yellowish from minute liver fragments. In all cases, it will be remembered, the conditioning fishes are fed in special containers and are carefully washed after feeding, so that in our experiments no food is placed directly in the food-conditioned water; it comes only from regurgitation or from faeces. When the concentrated conditioned water is diluted down to the strength used in the growth assays, the opalescence is largely lost.

The series of spectrographic analyses of the concentrated fish-conditioned water showed pictures similar to those obtained by direct aqueous extracts from food. There are, in addition, indications of a protein effect presumably caused by materials excreted from the surfaces of the conditioning fishes; this effect, however, is masked, so far as spectrographic analyses are concerned, by the food materials introduced by the fishes by regurgitation or from their faeces.

When the whole conditioned water was used as the growth-assay medium, we have direct evidence that the fishes may eat the strings of faeces. Usually the water was sieved so that all particles larger than about 40 $\mu$ were fragmented or removed. We know that gold-

fishes of the size used will take particles of charcoal, which had passed through one of the standard sieves, into their alimentary tract; presumably they will take food particles as well.

In addition to gross and microscopic inspection and to spectrographic analyses, the presence of organic matter in the conditioned water is demonstrated by appropriate tests for "required oxygen"; that is, of the oxygen required to oxidize, under standard conditions, the organic material present. The growth-promoting power of the water showed a slight, but significantly positive, correlation with the amount of organic matter so revealed. For the experiments at hand, $r = 0.066 \pm$ a standard error of 0.021. Values of over twice the standard error are usually considered to be statistically significant.

Such a positive correlation between the amount of required oxygen and growth does not necessarily prove that it is the food value of the conditioned water that is effective in promoting growth. The growth promotion may be the result of the presence of some other sort of growth stimulant which is not yet analyzed.

Better evidence that the added food is an important conditioning factor is found in the results obtained by using fasting conditioning fishes as compared either with accompanying controls or with fishes growing in water conditioned by fed fishes. When all the waters are similarly sieved, the assay fishes grow significantly more in the water conditioned by fishes that are fed daily than in water conditioned by fasting fishes. The increase in growth is not as great as would be expected on the basis of the increase in organic matter in the water. They also grow more in water conditioned by fasting fishes than in the untreated control waters.

The importance of food has been checked in another way. The conditioned water both from starved and from fed fishes has been filtered through one or two layers of close-grained filter papers. In the thirteen experiments on filtered "starved" and filtered "fed" conditioned water, growth was greater than in the controls, but not significantly so. In experiments that were made simultaneously, growth did not differ in the two kinds of waters, although the organic matter was almost four times as great in the water from the fed fishes. When all twenty-six of these experiments with filtered conditioned water are compared with their respective controls, there

is a greater growth of 0.36 units in the filtered water, with $P = 0.0027$ and hence significant statistically. This shows either that particles small enough to pass these filters are used as food, and so have significant growth-promoting powers even when present in small amounts, or that there is some other growth stimulant present over and above that possibly furnished by the food value of the filtered conditioned water.

The latter suggestion is further supported by the work with protein extracts from the surfaces of the goldfishes. These have definite growth-promoting powers in *solutions* diluted 1 to 400,000 or even 1 to 800,000. The required oxygen tests show that the organic matter present is approximately equal to that in the filtered conditioned water from fasting fishes. The growth-promoting power is, however, much greater than in such waters.

Further, preliminary experiments on treating fish-conditioned water with charcoal indicate that something is taken from the water by the charcoal which, when eluded, has growth-promoting power. It is not well to dwell too long on these charcoal experiments until more data are available; there are indications, however, that the growth-promoting power of the conditioned water so treated with charcoal is retained, though perhaps lessened. These findings are in accord with absorption spectra from such waters.

The whole amount of evidence demonstrates that a part of the effect of water conditioned by the presence of fed fishes on growth is to be accounted for by its supplemental food value. Food accessories aside, there are enough solid food particles present and apparently available to allow feeding to proceed much beyond the 2 hours allotted to the normal daily contact with supplied food. This is in keeping with the reduced effect of the conditioned water which has been observed when all assay fishes were fed a particularly favorable diet. The experiments do, however, suggest strongly that there is something more than a caloric food factor acting, the biochemical nature and the physiological effects of which await analysis.

With regard to the relative importance of these two aspects of growth promotion by homotypically conditioned water, it is too early to come to a definite conclusion. Perhaps we are unduly rash in even hazarding a guess. On the basis of the relation between the

amount of growth in water conditioned by fed fishes and that in water from fasting ones, and also by making a similar comparison of the growth of the former with that shown in filtered conditioned water, it would appear that under the conditions of our experiments food is some five or six times as effective as is any other growth-promoting factor which is possibly acting. Further experiments may even increase this estimate of the relative importance of the food value of the ordinary fish-conditioned water such as we have been using. When none of the particles have been removed by screening, the mean growth over that shown by accompanying controls is even greater. This is according to expectations if the amount of foodstuffs added by the conditioning fishes is, in fact, the principal growth-promoting factor in such waters.

With hypotonic waters (Allee and associates, 1934) and with certain sorts of toxic materials (Carpenter, 1927; Allee and Bowen, 1932), the conditioning fishes produce changes of other sorts which also make homotypically conditioned water a more favorable medium for fish growth than the untreated water would have been. These studies, together with those reported in the present paper, go far toward placing another phase of mass physiology on a sound experimental basis.

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# SELECTION OF FOOD IN PARAMECIUM TRICHIUM[1]

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THE ability of a ciliated protozoon to select its food is still an open question. Schaeffer (1910) described selection in *Stentor*. Day and Bentley (1911) state that *Paramecium* has a limited ability to learn, although these authors were not dealing with food. Metalnikow (1912) concludes that *Paramecium* can learn to select nutritious from innutritious particles by the induction of a condition analogous to a conditioned reflex. Hetherington (1934) states that *Colpidium* sometimes failed to ingest certain types of particles present in the medium which he used. On the other hand, Wladimirsky (according to Calkins, 1933) concludes that Protozoa cannot select nor learn to select food. Calkins (1933) admits selection in such discontinuously feeding ciliates as *Actinobolina* (=*Actinobolus*) but is skeptical of the ability of continuously feeding forms, such as *Paramecium*, to select food.

Among amoeboid forms the situation is clearer. Schaeffer (1916) concludes that *Amoeba proteus* ingests more flagellates, whereas *A. dubia* takes in a greater number of diatoms and desmids. He also (1917) believes *Amoeba* capable "of exercising very nice discrimination in feeding between two particles of different composition—one digestible, the other not. . . . ." Kepner and Whitlock (1920, p. 401) say, "The Ameba seems to have a marked preference for Chilomonas paramecium." Similarly, Mast and Hahnert (1935) conclude that *Amoeba proteus* feeds largely upon *Chilomonas* and *Colpidium*. However, these authors found that under certain circumstances this animal ingests almost any organism which is of appropriate size and is not too active, "but it does not ingest them indiscriminately, e.g., it rarely takes *Monas* if *Chilomonas* or *Colpidium* is available" (p. 270).

From a review of the literature it is apparent that there is practi-

[1] Thanks are due to Dr. B. R. Lutz, of Boston University, for furnishing space and facilities for the work upon which this paper is based.

cally unanimous agreement among workers with amoebae that these forms can and do select their food. On the other hand, no such agreement exists among workers with the ciliates, the greatest doubt centering upon the reactions of the continuously feeding forms, such as *Paramecium*. The following questions, therefore, need to be answered: Can any of the continuously feeding ciliates select their food? If so, do individuals within a species vary in this ability? Is there a similar interspecific variation within a genus? If we admit selection in any form, precisely in what sense do we use the term "selection"?

The present paper submits evidence bearing upon these questions. The principal organism used for experiment was *Paramecium trichium* Stokes, although *P. caudatum* was also used to a limited extent for comparative purposes. *Paramecium trichium* was chosen for study partly because it happened to be available but also because a search of the literature failed to reveal any work which had been done on the nutrition of this form and it was thought that interesting results might be obtained with a species closely related to *P. caudatum*, upon which much work has been done. Variation in the method of ingestion in these two species has already been found (Bragg, 1935*b*), and this fact suggests that variation in selective ability might also be shown.

Some of the confusion apparent from a review of the literature on this subject is probably due to the unqualified use of terms teleological in their implication, such as "choose" and "select." I think no one would attribute conscious selection of food to the Protozoa. The real question is: Do Protozoa ingest certain particles from the surrounding medium to the total or partial exclusion of others of comparable size and abundance but of a different nature? It is convenient to use the term "selection" for this type of phenomenon, and it is in this sense that the term is used in the present paper.

## MATERIALS AND METHODS

The animals used in these experiments were collected in Andover, Massachusetts, and were cultured in standard hay infusion at room temperatures. Both *Paramecium trichium* and *P. caudatum* were used, the latter species mostly for comparative purposes.

The method of observation was as follows: A small drop of the culture fluid containing the organisms was placed on a slide, a small amount of the substance to be fed was mixed with this, and a cover slip was sealed over the drop with vaseline. It is to be noted that at all times the animals had free access to their normal food supply, the bacteria in the culture. Observations were made as the animals became quiet about masses of zoöglea or moved slowly along the bottom. The rate of individual vacuole formation was taken with a stop watch and is, in each case, the time elapsing between the instant a given vacuole dropped from the pharynx and the instant the next one dropped.

Substances fed the animals were carmine, carmine mixed with neutral red, and haemoglobin powder. The carmine was that of Colman and Bell. The haemoglobin was that of Eimer and Amend. The neutral red was made up as recommended by Shapiro (1927), 0.5 mg. per 1 c.c. distilled water; but since, when used, the stain was mixed with the culture fluid, the exact strength of it reaching the animals is unknown.

## OBSERVATIONS

*Paramecium caudatum.*—Individuals of *P. caudatum* were found to vary greatly in the number of carmine-containing vacuoles which they would form in a given time. For example, after less than 5 minutes in one preparation one individual held twenty vacuoles containing carmine. Others beside it had one, two, three, or a few more. Carmine grains were continuously being shunted from the buccal grooves of all of the animals as I watched. After 25 minutes in this preparation some individuals had no carmine-containing vacuoles: others had up to thirteen. After 1 hour and 15 minutes, thirty-five animals, taken at random, averaged 14.83 vacuoles containing carmine. Of these, eleven individuals contained less than ten; fourteen contained from ten to nineteen; and ten had more than nineteen. One animal contained twenty-nine, the highest number recorded. All of these animals contained bacterial vacuoles of the usual type as well, many of which must have been formed while the carmine was present. These observations were repeated many times and always with comparable results. Therefore, it is concluded that *P.*

*caudatum* has a limited selective ability which is differentially distributed among individuals.

*Paramecium trichium.*—The results with *P. trichium* indicate that this species selects bacteria from carmine even more readily than does *P. caudatum.* When individuals are taken at random and the number of carmine-containing vacuoles is counted in each after various lengths of time, some individuals are seen to have no carmine-containing vacuoles. Others contain but a few of them. A few individuals have large numbers. Table I gives the observed data in a typical case. Further data are not given since they were all consistent with this.

TABLE I

NUMBER OF CARMINE-CONTAINING VACUOLES IN INDIVIDUALS OF *Paramecium trichium* AFTER VARIOUS LENGTHS OF TIME IN A SINGLE PREPARATION

| Time (Minutes) | Number of Vacuoles | Average |
|---|---|---|
| 2–12 | 3, 5, 2, 2, 1, 0, 0, 3, 2, 3, 3, 2, 0, 4, 3, 3, 3, 3, 3 | 2.37− |
| 20–30 | 0, 1, 1, 3, 2, 3, 4, 1, 7, 5, 3, 3, 2, 6, 4, 2, 3, 2, 4, 3, 2, 2 | 2.86+ |
| 30 | 10, 16, 0, 6, 10, 21, 9, 22, 11, 25, 12, 18 | 13.33+ |
| 35–45 | 3, 0, 3, 3, 2, 1, 1, 2, 2, 3, 1, 1, 1, 1, 4 | 1.87− |
| 75 | 1, 4, 4, 2, 3, 5, 6, 4, 0, 13, 1, 4, 3, 22, 0, 6, 2, 11, 2, 13, 1, 4, 10, 1, 11, 2, 6, 1, 15, 9, 2, 6, 5, 1 | 5.29+ |

These results may be interpreted in two different ways: either the animals, with some exceptions, took in very little carmine, or they threw it out about as fast as they took it in. The latter possibility has to be kept in mind, for I have seen a carmine-containing vacuole egested by *P. trichium* within 15 minutes of its formation. Furthermore, Metalnikow (1912) noted that in *P. caudatum* food vacuoles containing innutritious particles were retained for relatively short times in the body of the organism. The fact that there were so few animals which had large amounts of carmine in their bodies and the fact that streams of carmine grains could be seen being thrown out of the buccal grooves of the animals as they fed lead me to favor the first interpretation. In this connection it is also to be noted that all of these animals contained bacterial vacuoles having no carmine in them.

Greenwood (1886–87) studying *Amoeba,* suggested that when this

organism ingests carmine it does so incidently, the stimulus being supplied by a food organism in the immediate neighborhood. To test this matter in *Paramecium trichium*, individuals in a single preparation were taken and their carmine-containing vacuoles recorded in two sets, (1) those containing large masses of carmine as opposed to (2) those having no more than four grains. Table II shows the result in a typical case. Nearly 75 per cent of the vacuoles containing carmine held but a few grains each, and most of these were very small grains which could easily have been swept into the pharynx under the stimulus afforded by the bacteria without being perceived by the animal.

TABLE II

NUMBER OF VACUOLES CONTAINING LARGE AMOUNTS OF CARMINE AS COMPARED WITH THOSE CONTAINING NO MORE THAN FOUR GRAINS EACH IN TEN INDIVIDUALS OF *Paramecium trichium* AFTER ½ HOUR IN A SINGLE PREPARATION

| | Individuals | | | | | | | | | | Total | Percentage |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | C | D | E | F | G | H | I | J | | |
| Total vacuoles..... | 9 | 14 | 7 | 23 | 8 | 24 | 18 | 8 | 30 | 20 | 161 | 1.00 |
| Large amounts..... | 6 | 7 | 0 | 7 | 0 | 5 | 6 | 2 | 6 | 4 | 43 | 0 267+ |
| Small amounts..... | 3 | 7 | 7 | 16 | 8 | 19 | 12 | 6 | 24 | 16 | 118 | 0.732+ |

A further test was made by tabulating all of the food vacuoles within the animals, recording the number of vacuoles containing no carmine, those containing small amounts, as above, and those containing large masses. A typical case is shown in Table III. Of the ninety-three vacuoles reported in the table, approximately 48 per cent had small amounts of carmine, and only about 12 per cent contained large masses.

When neutral red and carmine were presented to the animals together, a most surprising result was obtained. Every vacuole now formed had large masses of carmine, and carmine grains could clearly be seen pouring down the buccal grooves of the animals and entering the vacuoles. Furthermore, relatively little carmine could be seen to be swept out of the buccal grooves of the feeding animals. I at first thought that perhaps the red color of the vacuoles due to the

dye was being confounded with the red color of the carmine grains. Long and careful observations, however, convinced me that this was not the case. The angular nature of the carmine grains could clearly be seen within the vacuoles.

TABLE III

NUMBER OF VACUOLES OF *Paramecium trichium* CONTAINING LARGE AMOUNTS OF CARMINE AS COMPARED WITH THOSE CONTAINING SMALL AMOUNTS OR NO CARMINE AFTER ½ HOUR IN A SINGLE PREPARATION

| | Individuals | | | | | Total No. Vacuoles | Percentage |
|---|---|---|---|---|---|---|---|
| | L | M | N | O | P | | |
| Total vacuoles | 24 | 22 | 11 | 20 | 16 | 93 | 1.00 |
| Large amounts | 3 | 6 | 2 | 0 | 0 | 11 | 0.118+ |
| Small amounts | 15 | 6 | 4 | 4 | 16 | 45 | 0.483+ |
| No carmine | 6 | 10 | 5 | 16 | 0 | 37 | 0.397+ |

The food vacuoles so formed were of larger size than usual, as judged by the eye. Also, the rate of their formation immediately decreased about 50 per cent. After from 12 to 48 hours in the preparation, the rate had again increased, until now it was greater than the initial rate. The figures of a typical case are given in Table IV.

TABLE IV

RATE OF FOOD-VACUOLE FORMATION IN *Paramecium trichium* AFTER VARIOUS LENGTHS OF TIME IN A NEUTRAL-RED-CARMINE PREPARATION AS COMPARED WITH THE RATE WHEN THE ANIMALS INGEST ONLY MATERIALS FROM THE CULTURE

| Conditions | No. of Vacuoles | Time (Seconds) | |
|---|---|---|---|
| | | Range | Average |
| Fed culture only | 30 | 11.8–70.0 | 25.1 |
| Carmine and neutral red, 10–60 minutes | 36 | 14.0–99.0 | 50.6 |
| Carmine and neutral red, 12–48 hours | 36 | 8.4–23.2 | 16.9 |

My records also show that neutral red had a steadying effect upon the time required for the formation of individual vacuoles; that is, the time taken by each vacuole to form tended to approach more

nearly to the average time taken for all vacuoles under the condition studied.

Limited data, given in Table V, do not show that neutral red has the same effect upon *Paramecium caudatum*. It should be noted, however, that this organism was not subjected to carmine and neutral red together.

TABLE V

RATES OF FOOD-VACUOLE FORMATION IN *Paramecium caudatum* UNDER VARIOUS CONDITIONS

| Conditions | No. of Readings | Average Time (Seconds) |
|---|---|---|
| Culture only | 56 | 41 1 |
| Carmine added | 35 | 44 4 |
| Neutral red added | 36 | 44 1 |

Haemoglobin powder was completely rejected by *P. trichium*. In a typical preparation, for example, the animals were under constant observation for 35 minutes and not a single haemoglobin-containing vacuole was seen. At the same time, many vacuoles containing bacteria were seen to form, and all of the animals were throwing haemoglobin granules from the buccal grooves. Several preparations containing varying quantities of haemoglobin and organisms from different cultures gave the same result.

When the animals were first placed into the haemoglobin preparations, many responded by avoiding reactions of the type described by Jennings (1931) and by eventually congregating in the region of the slide containing the least haemoglobin. After a time they became quiet here and continued to feed upon the bacteria in the culture. These facts suggest that either some small amount of a soluble substance was present as an impurity in the haemoglobin or that the haemoglobin itself was slightly soluble. In any case, the animals failed completely to ingest it.

In addition to the evidence already presented, another type of observation indicates that certain individuals of *Paramecium trichium* may show very marked selective action. On two occasions individuals were seen whose vacuoles contained large bodies some of which so closely resembled the flagellate *Chilomonas paramecium*,

which was abundant in the culture, that I had no hesitation in believing them to be this organism. The first of these animals was found in a haemoglobin preparation. It contained twenty exceptionally large food vacuoles, each containing a single *Chilomonas*. This specimen had no typical food vacuoles. The second case of this sort was seen the following day in an animal from the same culture from which the first had come. In this case nothing had been added to the culture. The animal had several normal food vacuoles containing bacteria and one large vacuole which held a *Chilomonas*. Careful search of all of my cultures failed to reveal other specimens of this type.

As showing selective action on the part of these paramecia, these observations are of some significance. The mouth of *Paramecium* is not large in any species of the genus, and we are here dealing with the smallest *Paramecium* yet described. Its length is given by Kudo (1931) as from 70 to 100 $\mu$, while that of *Chilomonas* is given as from 30 to 50 $\mu$. Assuming that we have the largest *Paramecium* and the smallest *Chilomonas* within these limits, it is still difficult to understand how the one could ingest the other without the mouth becoming considerably stretched; and the stretching of the mouth during ingestion, while quite common in normally carnivorous ciliates, has not been reported, so far as I am aware, in *Paramecium*. These flagellates must have been ingested, therefore, with considerable difficulty. These observations prove conclusively that at least some individuals of *P. trichium* are able to select their food. Such marked ability to select food organisms must be extremely rare, however, since I have watched thousands of food vacuoles form in *P. trichium*, *P. caudatum*, and *P. aurelia* and have never seen any indication of this sort of thing except as noted above.

It would be of extreme interest to observe a *Paramecium* ingesting so large an organism, and to compare the movements of the food vacuole with those normal to the organism, as recently described (Bragg, 1935*a*).

## SUMMARY

*Paramecium trichium* was fed carmine, carmine mixed with neutral red, and haemoglobin powder, by mixing each with the culture fluid containing the organisms. Observations showed that more food

vacuoles containing no carmine, or but a few grains of it, were formed than those holding large masses. Individuals varied considerably in this respect. Neutral red inhibited the selective action. It also had the effect of at first slowing the rate of vacuole formation and markedly increasing the size of individual vacuoles; later the vacuole formation rate was greatly increased. Haemoglobin was totally rejected by the animals. Two individuals were found which had ingested *Chilomonas*, one of them to the total exclusion of other materials.

It is concluded that *Paramecium trichium* has a limited ability to select its food, that individuals vary in the amount of selective action which they show, and that the mechanism through which the selection works involves the direction and probably the rate of beating of the peribuccal cilia.

More limited observations on *P. caudatum* confirm the conclusions of those who have maintained that this species is able to select its food. They also indicate that this species has less of this ability than has *P. trichium*. Incidently, this difference between these two species, when added to the difference already found in their ingestive mechanisms (Bragg, 1935*a*), may be of some importance to those interested in tracing nutritional conditions in other closely related species.

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# QUALITATIVE AND QUANTITATIVE CHANGES IN RADIOSENSITIVITY OF GRASSHOPPER EGGS DURING EARLY DEVELOPMENT[1, 2]

(Three figures, one plate)

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THE generalization of Bergonie and Tribondeau (1906), as it has been applied to embryology, indicates that as an organism develops it becomes steadily more resistant to radiation. There have been several recent papers which reveal certain instances when the embryo is more resistant, and certain others when the developing organism is more susceptible than the foregoing generalization would indicate. P. S. Henshaw (1932) and P. S. and C. T. Henshaw (1933) point out that gastrulation in the *Drosophila* egg is a particularly resistant stage to radiation capable of penetrating uniformly to all parts, and that this increased resistance may be related to the paucity of cells in active mitosis at this stage of development. Scott (1934) presents data on eggs of *Calliphora*, and he agrees with the results of the Henshaws (1933). The effects of irradiation appear very slowly, and some recovery is noted if eggs of the grasshopper are treated during the prolonged period of developmental inactivity (Evans, 1934). It appears from the foregoing reports, as well as from many others, that developmental inactivity lowers the susceptibility of organisms to Roentgen rays. However, the later papers of Henshaw and Francis (1933, 1936) indicate that the exact relationship is not clear.

Scott (1934) reports that in *Calliphora* the eggs in the stage just preceding the blastula and also preceding the gastrula are extremely

[1] The writer wishes to express his appreciation to Professors J. H. Bodine, H. D. Kerr, and E. Slifer for their advice and constructive criticism during the course of this investigation.

[2] Aided by a grant from the Committee on Radiations of the National Research Council and by the Rockefeller Foundation Fund for Research on Physiology of the Cell.

susceptible to irradiation. These increases in vulnerability are explained on the basis of the complexity of cell changes which must occur at these stages. Packard (1935) also finds that in *Drosophila* the eggs are particularly susceptible just before blastulation and during invagination, when they are undergoing physiologic and morphologic changes.

The investigation of changes in susceptibility of grasshopper (*Melanoplus differentialis*) eggs to Roentgen rays has been undertaken in the hope of making some contributions to the knowledge of mechanisms involved in ontogenetic development, as well as of learning more concerning the biological effects of Roentgen radiation. The development of the grasshopper egg may be divided into three stages: (1) pre-diapause, (2) diapause, (3) post-diapause. The pre-diapause development involves the formation and early differentiation of the embryo. The diapause is a period of developmental inactivity. During post-diapause development the embryo goes through blastokinesis, engulfs the yolk, and hatches. The reader is referred to the following papers for details concerning the morphological and physiological changes involved: Bodine (1932) and Slifer (1932).

## MATERIALS AND METHODS

The radiation was delivered at 130 kv., 5 ma., 30 cm. distance, and was filtered with 2-mm. cardboard. The dosage was applied in one treatment, and the amount was increased by lengthening the exposure time. The intensity was usually 200 roentgens per minute, measured in air.

The eggs were removed from the sand immediately after they had been laid, and were incubated at 25° C. They were irradiated at room temperature (approximately 26° C.) on moist filter paper on a strip of gauze suspended 3 feet above the floor to avoid scatter.

The early development of the grasshopper, *Melanoplus differentialis*, has been studied histologically by Slifer and King (1934). It is possible by the use of the foregoing paper (and from communication with Dr. Slifer) to apply the following chart relating the internal structure of the egg to its age at 25° C.:

Freshly laid eggs—first maturation division
9 hours—both polar bodies given off and from 1 to 3 nuclei present

1 day—from 61 to 32 segmentation nuclei present
2 days—many segmentation nuclei, some in peripheral protoplasm
3 days—definite germ band from one to two cell layers thick
4 days—germ band from two to four cell layers thick
5 days—invagination and formation of germinal layers

The embryo has definite form after 5 days and may even be dissected from the egg for closer observation. From this time on to the twenty-first day the embryo enlarges and differentiates steadily. The reader is referred to Slifer (1932) for the details of the morphological stages.

## EXPERIMENTAL

Figure 1 gives the results of a series of experiments in which eggs of different ages at 25° C. are exposed to dosages of 100, 200, 300,

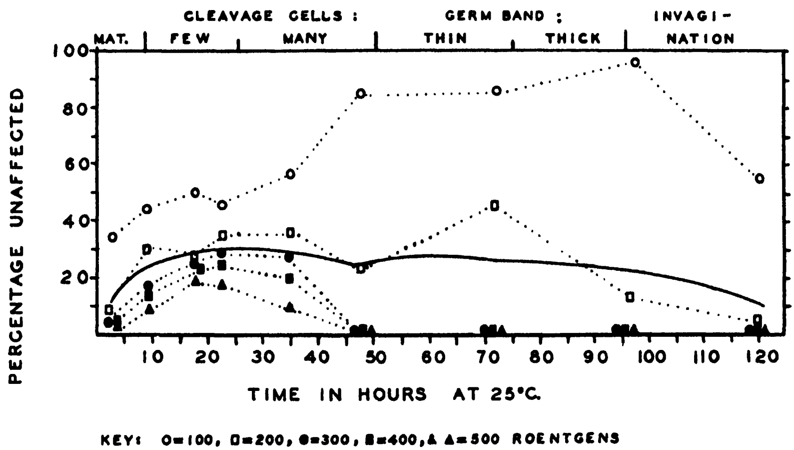



FIG. 1.—Graph showing changes in resistance to Roentgen rays during the first five days (at 25° C.) of development. The ordinates indicate number of eggs containing complete embryos, when examined at the time of diapause, in percentage of the controls. The abscissas show the time in hours. The condition of the egg at the time of irradiation is shown at the top of the graph. The heavy line represents the average resistance to all five of the dosages indicated in the key.

400, and 500 roentgens. Each symbol represents the resistance of eggs of an age to a dosage. The eggs are examined at the time of diapause. The controls at this stage are uniform, and any structural injuries of the irradiated embryos are easily recognized. The number of eggs in each lot varies from 42 to 122, and each symbol represents

the average of several lots. The solid line in the graph represents the average resistance to all five dosages and indicates in a general way the changes in susceptibility during the first five days of development. It may be seen from the graph that the general resistance is very low during the first 3 or 4 hours, but increases after the ninth hour and remains at about the same level until the fourth day. The resistance to 200 roentgens appears to follow about the same curve as the average of all five dosages, being lowest (after ninth hour) during the fifth day. It appears from an examination of the results of irradiation with 100 roentgens that the resistance is lower during the first two days than during the next two. The contrary seems to be the case following heavier irradiations (300, 400, and 500 roentgens). One way of expressing this change is to say that during the first two days a few of the eggs are extremely susceptible and a few are extremely resistant, whereas during the next two days the resistance of all the eggs is about the same and the effective dosage is 300 roentgens, or above.

The above-mentioned graph expresses the variation in only one end-point, namely, the percentage of eggs uninjured when examined at the time of diapause. The variation in the character of the injury, as well as the frequency of its occurrence, is shown in Figure 2. This figure consists of twelve graphs, representing the relation of 100, 200, 300, 400, and 500 roentgens to the extent of injury, and the percentage of its occurrence in eggs from 1 to 13 days of age. The percentage of eggs completely unaffected is indicated by the height of the solid black columns. On the first day the general resistance is not high, but a few eggs are not affected by even 500 roentgens. After the second day of development all eggs are susceptible to more than 300 roentgens, and this condition remains until the eighth day. All eggs are injured by dosages above 400 roentgens up to the tenth day, and all are affected by more than 500 roentgens until the eleventh day. This would seem to indicate that on the first day a few eggs are extremely resistant; and again, beginning with the eighth day, more resistant eggs are found. The height of the solid columns gives the percentage of resistance to the various dosages. It will be seen that on the second, third, and fourth days the resistance to the lower dosages increases markedly and then drops on the

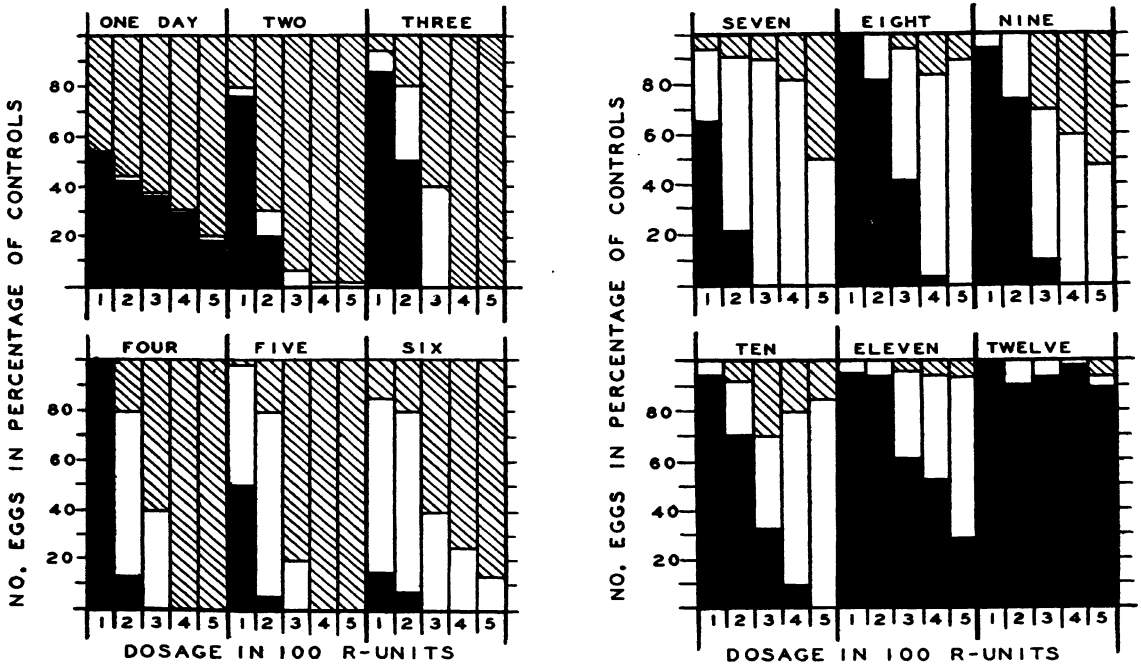



FIG. 2.—Twelve block graphs indicating the results of a series of experiments in which eggs of different ages were irradiated and the extent of injury to individual embryos determined at the time of diapause. Incubation was at 25° C. The solid blocks indicate the number of eggs containing complete embryos, in percentage of controls. The shaded blocks indicate the percentage of eggs which contained no embryos at all. The unshaded areas represent the percentage of eggs in which the embryos were partially injured. The age of the eggs (in days) in each experiment is shown at the top of the graph. The dosages are indicatted at the base, in terms of 100 roentgens.

fifth and sixth days. It appears that on these two days (fifth and sixth) all of the eggs are particularly susceptible to even small dosages. Beginning with the seventh day the resistance to the lower dosages begins to pick up again.

Let us now consider the extent of injury to the individuals at the various stages. The shaded areas indicate the percentage of eggs which contain no embryos at all when examined at the time of diapause. The blank areas represent the percentage of eggs which contained partially injured embryos when examined. It will be seen that on the first and second days the irradiation either destroys the entire embryo or it apparently does not affect it at all. Beginning with the third day, some differentiation has evidently taken place, in that cells are now differentially susceptible. The number of partially injured embryos increases with the dosage, and with ageing more and more of the affected eggs contain embryos only partially destroyed. Eggs which had been irradiated on the third and fourth days, when examined at the time of diapause, contain many embryos with the whole head, thorax, or abdomen missing. After the fourth day, the irradiation results in shrinking and histolysis of parts, but the injury is more localized. As the dosage is increased, the injury is more extensive. In summary, the graphs in Figure 2 show that the effects of roentgen irradiation parallel to a certain extent the progress of differentiation. With the onset of differentiation the parts of the organisms become differentially susceptible. In general, as the embryos become more differentiated, they are more resistant to light dosages; but there is one stage during the fifth and sixth days where the eggs are all very susceptible. This increased susceptibility is apparently related to the histological picture, which shows that at this time the embryo is in the process of invagination and formation of the rudimentary structures of the embryo (Slifer, unpublished). This finding indicates that in the grasshopper the resistance of the embryo does not always increase steadily, and in stages involving extensive differentiation of tissues it is unusually susceptible.

Another indication that this period of increased sensitivity is related to differentiation is the appearance of abnormal structures in embryos irradiated during this period. Figure 3 shows typical morphological aberrations that are found in the irradiated eggs examined

some time after the onset of diapause. (No. 1 is a drawing of a control at the time of diapause.) At the present time the writer is unable to correlate exactly either dosage or time of irradiation with a particular type or degree of abnormality, although certain trends are evident. The production of abnormalities is most frequent during the fifth, sixth, and seventh days. Only 5 eggs out of 1,527 irradiated between the first and fourth days produced abnormalities. One hundred and thirty-four out of 1,176 rayed between the ages of 5 and

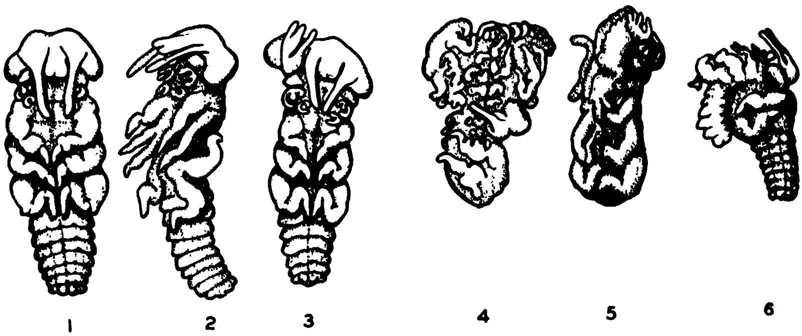



FIG. 3.—Drawings of embryos showing some of the anomalies found in embryos irradiated in the five- to six-day stages. No. 1 is a ventral view of a control at the time of diapause. No. 2 is an irradiated embryo (same view, same scale) showing bifurcation of right mesothoracic leg and of left metathoracic. No. 3, another experimental with small aberrant growth protruding from right side of head. No. 4, disintegration of original abdomen, and enormous secondary growth from head region apparently in three sets of appendage-bearing somites. No. 5, anterior outgrowth from head resembling a secondary embryo. No. 6, complete embryo occupying region of original head and mouth parts.

8 days resulted in anomalies. Out of 1,575 eggs rayed between the ages of 8 and 13 days, not one exhibited anomalies such as indicated above. It therefore appears that the production of the anomalies may be related to invagination and the formation of the basic structures which are in process during the fifth, sixth, and seventh days.

It was noticed that the serosa and cuticle are present in many of the irradiated eggs at diapause even though the embryo has been completely destroyed. It is necessary to expose eggs of 1 day of age to 1,000 roentgens in order to prevent the formation of serosa and cuticle. Even this dosage fails to prevent their formation when 3-day eggs are treated, although the cuticle is not visible until several

days later. This dosage (1,000 roentgens) is twice that necessary to inhibit the formation of the embryo; so these cells that do not go into the formation of the embryo proper must differentiate early and are apparently very resistant.

Several groups of eggs were irradiated on the first and second days of development with the micropyle end of the egg containing segmentation nuclei and surrounding protoplasm) protected with lead. All of these eggs contained complete embryos when examined, at the time of diapause, even following an irradiation of 1,000 roentgens. When the micropyle end of the egg is uncovered and the other half of the egg protected with lead, the results are the same (complete inhibition of embryo formation) as when the whole egg was irradiated. It appears, therefore, that the nuclei are developmental centers and that nothing is contained in the yolk at this time which, when irradiated (700–1,000 roentgens) affects the formation of the pre-diapause embryo.

The procedure of partially protecting the egg was extended to later stages when the embryo is definitely formed. Pin holes were punched in a sheet of lead and the eggs were fastened to the lower surface, permitting certain regions to be exposed to the beam of radiation. A series of 5- and 6-day eggs was irradiated with the following regions exposed: (1) just the micropyle, (2) region just nearer the middle of the long axis of the egg, and (3) center of egg. Typical results are shown in *B* and *C* of Plate I. Some eggs that had been kept at 5° C. long enough to permit immediate post-diapause development at 25° C. were also partially protected during irradiation. The disintegration appears only in regions exposed; but in cases where the region is as extensive as those shown in 1, 2, 3, of *E*, Plate I, the organisms do not go beyond the first few days of post-diapause development. In a few cases the injury was localized to the mouth parts, and such embryos hatch but die within a few days. These experiments serve chiefly to show some possibilities of applying this method to a study of the mechanics of early development.

## DISCUSSION

It is difficult to compare the changes in susceptibility of the eggs of the grasshopper with those reported for *Calliphora* (Scott, 1934)

and for *Drosophila* (Henshaw P. S., 1932; P. S. and C. T. Henshaw, 1933; Packard, 1935). The comparison is made unreliable because of the differences in the development of the organisms and the experimental methods employed. It is apparent, however, that all agree that increases in susceptibility are related to (1) cellular activity and (2) complex cellular differentiation.

The finding that the resistance of grasshopper eggs to a dosage of 100 roentgens increases with development during the first four days agrees with the conception of Bergonie and Tribondeau (1906). The fact that 3–4-day eggs are partially affected by dosages of 300–500 roentgens, whereas at least a few of the earlier (1–2-day) eggs are apparently unaffected by these dosages, appears to be inconsistent. There is a possible assumption, however, which will make this apparent exception more in accord with what one would expect. This assumption is that unaffected cells of the 1–2-day stage are able to take the place of the ones destroyed by the irradiation. As differentiation progresses, the number of these totipotent cells naturally decreases.

The increased susceptibility during the fifth, sixth, and seventh days may be explained as a peculiar interplay of cell susceptibility and regenerative forces. The morphological changes at this time indicate that an extensive differentiation of the embryo is in progress. The increased vulnerability to even low dosages may be due to the complexity of cell changes involved, as is suggested by Scott (1934) and by Packard (1935), and may be complicated by changes in the regenerative power of certain cells. The secondary growths of embryos irradiated at this stage indicate the presence of cells possessing the powers of renewed growth even though the proper differentiation may be upset. There seems to be no evidence at the present as to whether the "regeneration" is related to the presence of certain cells which have retained their early embryonic structure and power of further differentiation or whether advanced cells dedifferentiate and reform other types of cells.

The general increase in resistance as the embryos develop may be explained by either of two implications of the law of Bergonie and Tribondeau (1906). The first is the one usually referred to; and it states, in substance, that the resistance of an embryo is (1) propor-

## PLATE I

Photomicrographs of grasshopper embryos showing results of partially protecting eggs from irradiation.

*A*. Controls on the sixth to seventh days (25° C.)

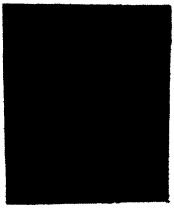
*B*. Results of irradiating (1,000 roentgens) center of six-day eggs. *X* is irradiated embryo showing abdomen to be missing. Other two embryos are controls at the time of diapause.

*C*. Embryos from eggs irradiated on the seventh to eighth days with only the micropyle exposed. Note injury to head region.

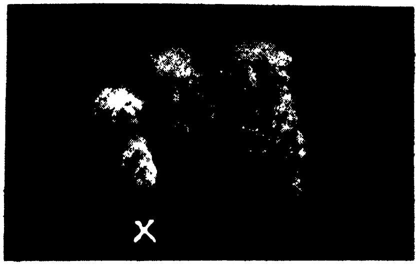
*D*. Controls after two months at 5° C. (in seventh to eighth day stages) are now capable of uninterrupted development at 25° C. until hatching.

*E*. Results of irradiating (1,000 roentgens) eggs similar to those of *D* with organisms partially protected with lead. No. 1 is an embryo taken from an egg irradiated with micropyle region protected. Nos. 3 and 4 are from eggs irradiated with micropyle region exposed. No. 4 is a control at the time these eggs were examined.

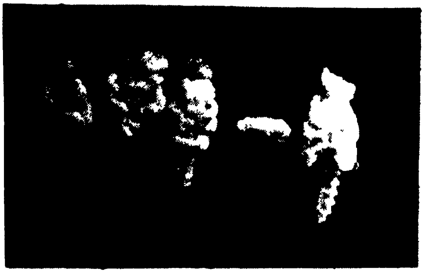
PLATE I



A




B



C

D


E

tional to its degree of differentiation and (2) inversely proportional to its potentiality for further development. The second viewpoint, which the writer wishes to emphasize, is the "potentiality of further development." It is generally observed that injury may not appear for some time following irradiation, and that it may be made to appear much sooner by increasing the dosage (Evans, 1934). Also, irradiated organisms which do not appear to be injured as long as they are developmentally inactive suddenly exhibit harmful effects on the initiation of development. It therefore becomes apparent that increased resistance of older organisms may be observed because they are nearer the time of the end-points employed and have not had the time or opportunity to express the full extent of the injury.

## CONCLUSIONS

The following conclusions are based on the results of experiments in which grasshopper eggs of different ages are irradiated and examined at the time of diapause:

1. During the first two days a few eggs are killed by low dosages, but some are resistant to even 500 roentgens. When the irradiated eggs are examined, they contain either a complete embryo or none at all.

2. The resistance to low dosages increases during the second and third days, but the number of complete embryos found after irradiations of 300–500 roentgens drops markedly. The embryos are not completely missing in the affected eggs. However, the injury is still extensive, and missing parts relate to a whole abdomen, thorax, set of mouth parts, or head.

3. From the third to the fifth day the germ band is thickening and the serosa is formed from the blastoderm. Irradiation at this time affects all of the embryos if it is of more than 300 roentgens. If it is less than 200 roentgens, hardly any of the eggs are affected. The injury at this stage is localized, chiefly to the more rapidly growing parts; but if the dosage is higher, the growth of the whole body is retarded.

4. The embryos are undergoing extensive changes in differentiation between the fifth and seventh days, and this stage is peculiarly sensitive to Roentgen rays. Although nearly all eggs are affected by even light dosages, certain regions possess the ability to develop more growing tissue, although the correct differentiation of this new tissue may be affected.

5. Following these extensive differentiation changes, the embryos become more resistant, and the effect of increasing the irradiation is evidenced by more extensive injury to individuals.

6. The foregoing conclusions, together with some preliminary experiments of another nature, indicate that the law of Bergonie and Tribondeau is not sufficient to explain all changes in susceptibility during development. However, it has implications which are strengthened by the foregoing findings, because the two factors which it recognizes (growth-rate and differentiation) are involved in even the detailed changes noted above.

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# OXYGEN CONSUMPTION AND RATES OF DEHYDRATION OF GRASSHOPPER EGGS (ORTHOPTERA)[1]

(Ten figures)

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INVESTIGATIONS regarding the effect of the removal of water from living materials may be classified into three general categories: first, the effect of desiccation on behavior of animals; second, the vital limit of desiccation and the rate of water loss; third, the effect of dehydration on physiological processes, such as respiration, etc.

In the first of these, results have, in general, shown an increased irritability as the first response of animals to desiccation (Shelford, 1913; Weese, 1917; Hamilton, 1917; Chenoweth, 1917; Hayes, 1927; and others).

Hall (1922) and Ludwig (1936) have reviewed the literature concerning the vital limits of desiccation; and from this, one is impressed by the great variability among organisms in their ability to withstand water loss. The rate of water loss is also an important factor. Durig (1901), for example, observed on the common European frog that a vital limit of desiccation was reached at 15 per cent weight loss, if water loss was rapid, while 30–39 per cent of the initial weight could be lost if the drying was slower. Gunn (1933) observed the same for the cockroach (*Blatta orientalis*), 44 per cent weight loss being the limit for slow and 32 per cent for rapid desiccation.

The rate of water loss by the same animal at different stages of development has been studied by relatively few workers. Zavadovskii (1932) found larvae of Trichostronhylidae became increasingly resistant to drying as they became older. Ludwig (1936), using the Japanese beetle (*Popillia japonica*), reports an increasing resistance

[1] Aided by a grant from the Rockefeller Foundation for work on the physiology of the normal cell.

to desiccation in the first, second, and third instar stages, with the pupae being the most resistant.

Both increases (Warburg, 1909; Caldwell, 1925; Hess, 1930; Buchanan, 1931; Duryee, 1932) and decreases (Loeb and Wasteneys, 1913; Inman, 1920; Caldwell, 1925; Bodine, 1933) in respiratory rates have been reported for animals when exposed to dehydrating hypertonic solutions.

In the present investigation the rates of dehydration and the resultant effects on the oxygen consumption of the eggs of the grasshopper, *Melanoplus differentialis*, have been studied.

## MATERIALS AND METHODS

The embryology of this species has been closely followed by Slifer (1932*a*), while the changes in respiratory rate during development have been recently pointed out by Boell (1935). Briefly, at 25° C. the embryo develops regularly to the twentieth or twenty-first day, at which time a period of non-development (diapause) follows. During diapause, mitosis ceases and respiration and other activities are markedly lowered or depressed. If such non-developing eggs are placed at low temperatures (0° C.) for several months, and subsequently returned to 25° C., development is resumed, and hatching occurs about 20 days later.

Two methods were employed for dehydration: a hypertonic balanced salt solution (Belar) (Slifer, 1934) and a calcium chloride desiccator. The solution used was ten times isotonic strength, and will be known hereafter as a 10 N Belar. The eggs were not immersed directly in the solution, since in non-aërated or non-shaken solutions they soon deplete the oxygen supply and development ceases.[2] Eggs were therefore covered with a thin film of the solution in question and placed in a glass-covered vessel on filter paper saturated with the hypertonic medium. Both the walls and top of the vessel were lined with filter paper, wet with the same 10 N Belar; and the eggs were changed to a new vessel daily to prevent undue concentration by evaporation. Further details of dehydration methods appear in what follows.

The degree of water loss was determined by weighing the eggs on a chainomatic balance, sensitive to 0.1 mg. The water content of

[2] Unpublished observations.

similar eggs during development has been followed and discussed by Bodine (1929).

Oxygen consumption was determined by means of differential Barcroft micromanometers, using individual eggs in all cases.

## RESULTS

### THE EFFECT OF DEHYDRATION ON OXYGEN-CONSUMPTION RATE

Figure 1 represents graphically the oxygen consumption for postdiapause eggs[3] dehydrated in 10 N Belar to the indicated amounts.

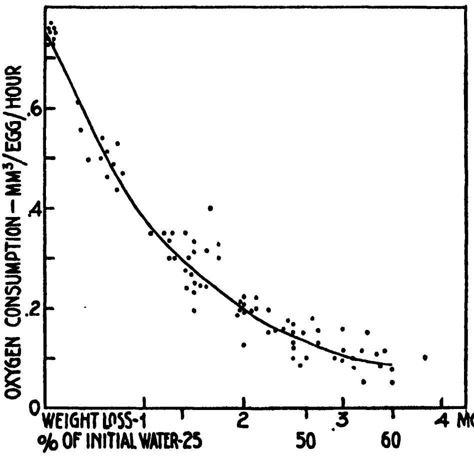


FIG. 1.—Oxygen consumption (cubic millimeters per egg per hour) of *Melanoplus differentialis* eggs at various levels of water loss (milligrams of weight loss and percentage of initial water). Each dot represents results for an individual egg. Temperature = 25° C.

From an inspection of this figure it will be noted that the rate of oxygen consumption drops rapidly during the first stages of water loss, tending to level off at about one-fifth of the normal undehydrated rate. This agrees with the previous results reported by Bodine (1933).

The effect of water recovery on the oxygen-consumption rates of dehydrated eggs is shown graphically for typical examples in Figure 2. If the water loss has not been excessive, recovery is slow and grad-

[3] "Postdiapause eggs" will be designated as those eggs in which the embryo has reached a stage in development about 12 days before hatching. The eyes are about midway between the two ends of the egg (Slifer, 1932*b*).

ual, both of water and of respiration (curves *A*, *B*, and *C*). If, however, water loss has proceeded beyond 2.5–3.0 mg., the egg gains water far in excess of that lost, sometimes regaining as much as 4 mg. above the initial weight. In such eggs, which occasionally swell enough to rupture the egg membranes, a "burst" of oxygen consumption invariably occurs (curves *D* and *E*). This "burst" sometimes reaches a height of 700 per cent of normal respiration, but it gradually drops off in a few days. In such eggs embryos are rarely found

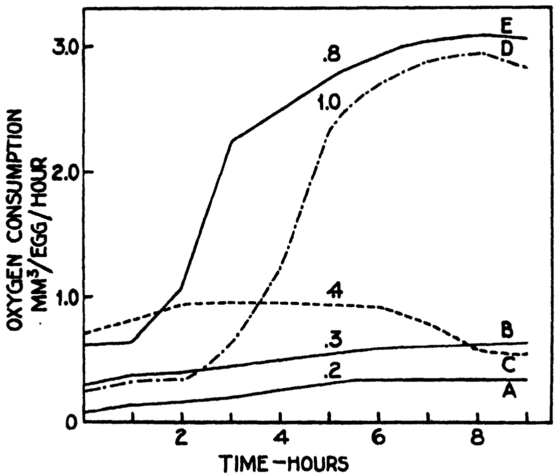


FIG. 2.—Effect of water recovery on oxygen-consumption rate of dehydrated postdiapause eggs. Oxygen consumption (cubic millimeters per egg per hour) plotted against time (hours). Number on curves indicates increase in weight (milligrams) above initial weight. Curves *A*, *B*, and *C*: individual eggs which lost 1.5–2.0 mg. during dehydration; *D* and *E*: eggs dehydrated 3.5 mg.

after swelling, although normal embryos were present at the beginning of the experiment, and shrunken but still visible embryos could be detected immediately after dehydration.

INDIVIDUAL VARIATION IN RATE OF DEHYDRATION

A large amount of variation was found in the rate at which water was lost. For example, in postdiapause eggs, after 24 hours' desiccation over $CaCl_2$, some eggs had not changed in weight, while others had lost as much as 82 per cent. Figure 3*P* shows graphically the extent of this variation. The eggs which fell into certain ranges in

respect to water (weight) loss are grouped together. Each egg was weighed, dried in the desiccator for 24 hours, and then re-weighed. Two per cent were found to have lost no weight; 11 per cent lost 0–0.5 mg.; 20 per cent lost 0.5–1.0 mg.; etc. One egg lost nearly 5 mg. It is interesting to compare this group with the diapause lot

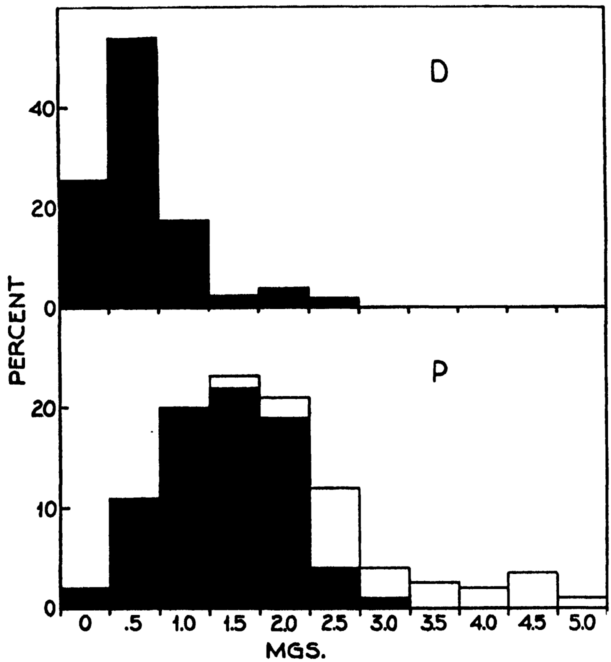


FIG. 3.—Variation in amount of weight decrease (desiccation) of diapause (*D*) and postdiapause (*P*) eggs exposed for 24 hours in $CaCL_2$ desiccator. Eggs, after exposure, were grouped as to weight loss: 0 mg., 0.0–0.5 mg., 0.5–1.0 mg., etc., and number in each group expressed as percentage of total number (300). Unshaded areas represent non-recovery.

(Fig. 3*D*). After the same length of drying time, 25 per cent of diapause eggs had lost no water; 54 per cent 0.0–0.5 mg.; etc. One can readily see that, while nearly all of the diapause eggs (80 per cent) had lost less than 0.5 mg. during the 24 hours' desiccation, 87 per cent of the postdiapause eggs had lost over 0.5 mg. during the same time. Although this is a wide spread, in the individuals of the older group (postdiapause) in particular, the rate of desiccation is more

rapid than in the diapause eggs. It should be mentioned that the postdiapause eggs are the most variable of any age, and that the diapause ones are the most uniform in regard to their individual differences in this respect.

This wide variation of water loss among individuals has been noted in other forms. Gunn (1933), using the cockroach, *Blatta orientalis*, found that from 5.5 to 32 per cent of the initial body weight could be lost in one day, even though the animals were subjected to the same environmental conditions. Ludwig (1936) mentions that certain larvae of the Japanese beetle are rapidly desiccated while others lose water very slowly. Larvae in the first instar may require from 23 to 142 hours to lose about 52 per cent of their weight.

### THE VITAL LIMIT OF DESICCATION

After the eggs (Fig. 3) were weighed, they were placed on filter paper, moistened with water, for 2 days. Each was then examined to determine which had survived, as indicated by yolk engulfment. The black shaded portion (Fig. 3) indicates those which were developing, while those not recovering are shown by the unshaded part. Nearly all recovered if the loss was less than 2 mg., or 33 per cent of the weight. In the group which lost 2.0–2.5 mg., only one-third recovered; and it was apparently impossible for those which had lost more than 2.5 mg. to proceed with development. This is about 46 per cent of the total initial water content.

### RATES OF DEHYDRATION

Figure 4 represents graphically the percentage of initial water content lost plotted against time of exposure to the 10 N Belar. Four indicated stages were used: 5- and 15-day prediapause, diapause, and postdiapause. Eggs of approximately the same weight were selected for each group. The temperature was kept at 25° C. Each curve is the mean of the dehydration curves for 300 eggs. The drying caused by the weighing had no appreciable effect on the rate of dehydration, as was demonstrated by weighing a group of postdiapause eggs every third day and noting that their water-loss rate did not vary significantly from that of the eggs weighed daily.

In terms of percentage of initial water lost, the developing eggs showed an increased rate of dehydration with increasing age. The

slowest group was the diapause lot. The limit of dehydration by 10 N Belar is about the same for all groups: 50–55 per cent of the initial water. This limit was ultimately reached by the diapause eggs, after about 6 weeks' exposure to the hypertonic solution.

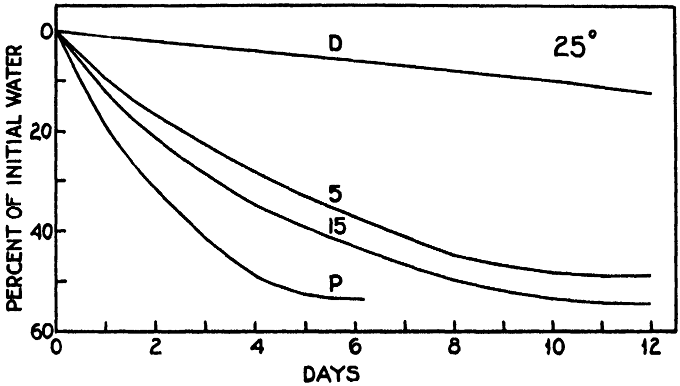


FIG. 4.—Percentage of initial water lost by eggs exposed to 10 N Belar. Temperature = 25° C. *5*, 5-day-old eggs. *15*, 15-day-old eggs. *D*, diapause eggs. *P*, postdiapause eggs.

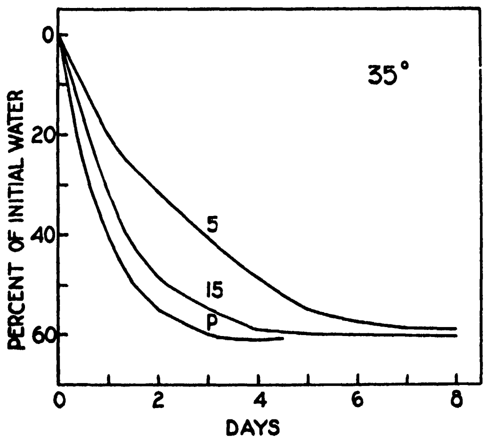


FIG. 5.—Same as Figure 4 except for temperature = 35° C

At 35° C. (Fig. 5), the rate of dehydration for all ages is higher, and the limit reached is about 60 per cent of the initial water. The curve for the rate of water loss of the diapause eggs is not shown since it followed almost exactly that for the postdiapause ones at

this temperature. It should be observed that eggs kept at temperatures as high as 35° C. have no apparent diapause.

The effect of three different temperatures on the rate of water loss by postdiapause eggs exposed to 10 N Belar is shown graphically in Figure 6. Since these eggs were all at the same morphological stage, they may be compared on the basis of actual weight loss. The 5° C. lot was eventually dehydrated to nearly the same level as the other groups. The $Q_{10}$ for the dehydration rate between the interval 25°–35° C. is about 2; and between 5° and 25° C. it is much higher. This

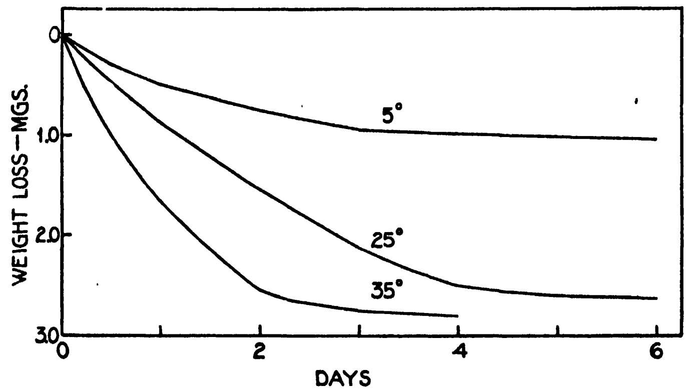


FIG. 6.—Weight loss (milligrams) of postdiapause eggs exposed to 10 N Belar at different temperatures, plotted against time (days).

type of variation in $Q_{10}$ values is the usual result found when dealing with biological processes, but would be unusual for a process which might be considered as depending entirely on physical forces.

Figure 7 represents graphically the rate of drying of eggs over $CaCl_2$. From an inspection of this figure it is obvious that no qualitative difference exists between the results obtained by this dry desiccation and the wet (10 N Belar) method. Four- and 15-day eggs were quite close together in their rate of water loss; postdiapause ones were the most rapid. Diapause eggs were again the most resistant, but eventually (after about 40 days) lost 73 per cent of their initial water. The other groups lost between 70 and 76 per cent.

Obviously, in both methods of dehydration a great difference is shown between the rate of water loss from diapause and postdia-

pause eggs at 25° C. At 35° C., however, this difference disappears. Diapause embryos at that temperature become, developmentally, postdiapause embryos. Now, at 0° C., so far as can be detected, all development ceases. A comparison of the rate of desiccation at this temperature of the two types of eggs is shown in Fig. 8. No significant difference in the actual amounts of water lost (Fig. 8*B*) can be found at this temperature, where all demonstrable activity in both types of eggs is reduced to zero. This would seem to indicate

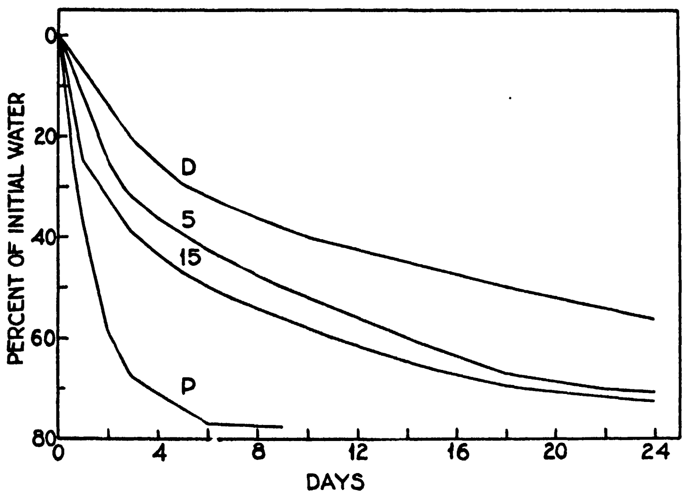


FIG. 7.—Percentage of initial water lost by exposure of eggs to desiccation over $CaCl_2$ plotted against time (days). Notations as in Figure 4. Temperature = 25° C.

that the difference in ability of the two types of eggs to withstand desiccation at developmental temperatures is related to the activity of the embryo. Accordingly, the rate of dehydration was determined for the isolated embryos. The methods used were those developed by Slifer (1934). Embryos of known stages were dissected out of the egg in isotonic Belar. After measuring the length of each embryo by means of an ocular micrometer, they were changed to 5 N Belar and measured every 5 minutes. Slifer (1934) found that postdiapause embryos in 5 N Belar shrunk to about 80 per cent of their initial length. This has been confirmed in the present observations (Fig. 9*P*). For diapause embryos (Fig. 9*D*), the same shrinkage occurs,

the rate of shrinking, if anything, being slightly more rapid than for the postdiapause embryos (Fig. 9*P*). Intact diapause eggs, therefore, are quite resistant to dehydration, while naked diapause embryos lose water just as rapidly as do diapause ones.

By centrifuging the grasshopper egg with the Beams ultracentrifuge, stratification of the contents occurs. The yolk, proteins, and remains of the embryo are clumped at one end of the egg, the remainder being filled with a clear, watery layer (Bodine and Boell,

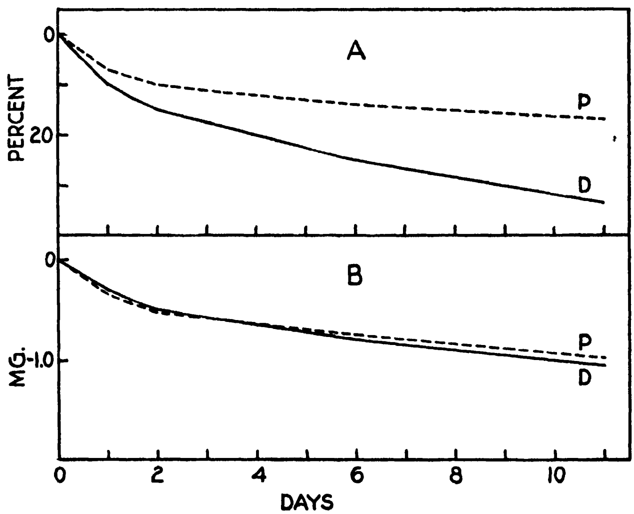


FIG. 8.—Percentage of initial water (*A*) and actual weight lost (*B*) by diapause (*D*) and postdiapause (*P*) eggs over $CaCl_2$ at 0° C. plotted against time (days).

1936). Eggs thus stratified have been employed to determine the rôle, if any, which the embryo itself plays in regulation of water loss by dehydration of the whole egg. The curves (Fig. 10*A*) still show the same qualitative rate difference between diapause and postdiapause eggs.

It has been shown that the respiration of centrifuged postdiapause eggs is depressed to a level approaching that of the diapause eggs. On the other hand, diapause eggs, treated in the same manner, respire at nearly their normal rate (Bodine and Boell, 1936). If the relationship between rate of desiccation and metabolic activity (Mellanby, 1932; Ludwig, 1936) is a simple function of respiratory

rate, the speed of desiccation of these centrifuged postdiapause eggs should then approach that of the diapause. Such, however, was not found to be the case.

Further evidence supporting the foregoing may be obtained by using intact eggs (if objection may be advanced to the centrifuge experiment on the grounds that such treatment is finally lethal). If postdiapause eggs be exposed to a mixture of 95 per cent carbon monoxide plus 5 per cent oxygen, respiration is depressed. Oxygen consumption of diapause eggs in the same gas mixture is little

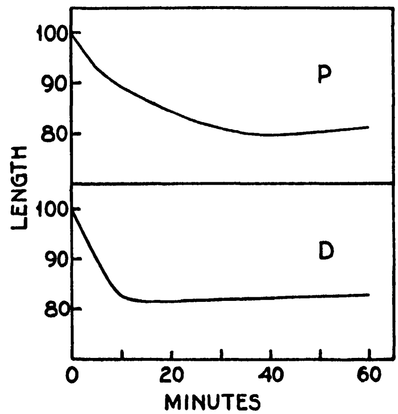


FIG. 9.—Changes in length (percentage) of isolated diapause (*D*) and postdiapause (*P*) embryos in 5 N Belar plotted against time (minutes). Each curve represents mean of 25 individuals.

affected (Bodine and Boell, 1934). Eggs of the two stages were placed in a $CaCl_2$ desiccator, which was then filled with this $CO/O_2$ mixture. The rate of water loss for each group was identical with that found in air.

The membranes surrounding the grasshopper egg are collectively known as the chorion and cuticle. The chorion may be removed from the egg after the cuticle has been formed (Slifer, 1932*b*). It was noticed that the chorions of postdiapause eggs were often cracked, sometimes peeling completely off the egg. In diapause and prediapause eggs the chorion is firmer—at least cracks or peelings are not often apparent. In order to test the function of the chorion in water loss, chorions were removed from a number of diapause and post-

diapause eggs and the eggs then dried as before (Fig. 10*b*). A qualitative difference in rate still exists between the two groups, although it is not quantitatively as large as for eggs with chorions untouched.

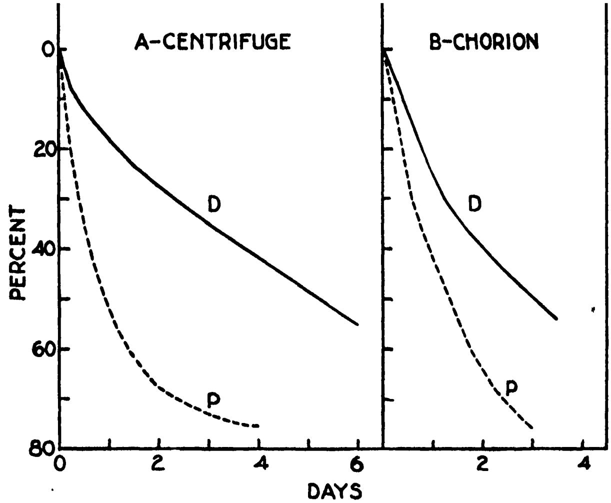


FIG. 10*A*.—Percentage of initial water lost by centrifuged diapause (*D*) and postdiapause (*P*) eggs plotted against time (days).

FIG. 10*B*.—Effect of removal of chorion on rate of water loss by diapause (*D*) and postdiapause (*P*) eggs.

Undoubtedly, the chorion serves for some protection, but it alone cannot account for the differences in water-loss rates of the two types of eggs.

## DISCUSSION

It is not possible to explain the varying rates of water loss on the basis of stretching of the egg membranes. The egg gradually becomes heavier during its development, and this increase in weight is undoubtedly due to water intake (Bodine, 1929). It is rapid during prediapause, slowing down about the fifteenth to twentieth day of development. Weight remains relatively constant during diapause, increasing very slowly, if at all. Postdiapause eggs take up large quantities of water, about 2 mg. being the average increase in weight over a diapause egg. It is during these periods while water is normally being taken into the egg that it is lost most readily under

experimental dehydrating conditions. Diapause eggs, on the other hand, remain relatively constant in weight, take in but little water, and are very resistant to water loss. During the periods in which water is being imbibed, the egg increases in size and the membranes become stretched. Sometimes they are unable to stand so much swelling and rupture. The egg membranes, however, are not perfectly elastic, as is evident from the fact that postdiapause eggs commence to appear shrunken when they have lost 2 mg. or less of water. Diapause eggs, which contain about 2 mg. less water than postdiapause ones, are perfectly turgid; and such stretched membranes are undoubtedly thinner, as demonstrated by Jahn's (1936) work on capacitance. Being thinner, they may lose water more rapidly than before; and this might possibly explain the observation that 5-day, 15-day, and postdiapause eggs are successively less resistant to desiccation. Each group has respectively taken up more water, resulting in stretched and thinner membranes; but the diapause group, on the other hand, shows a striking exception. Here the water content is just as high, the membranes are stretched just as tight, and are as thin as in the 15-day group; but the rate of water loss is lower than for any of the developing groups. Furthermore, when diapause and postdiapause eggs are placed at 0° C., the rate of water loss in terms of actual weight loss is almost identical. In terms of percentage of initial water, the postdiapause egg is dehydrated at a slower rate than the diapause one—and this despite the fact that the postdiapause egg has more water to lose.

An extensive investigation of the properties of the membranes of the egg has recently been made by Jahn (1935*a*, 1935*b*, 1936). The only change in ionic permeability demonstrable by resistance measurements occurs with the laying-down of the cuticle, which takes place about the seventh day. No change was observed during diapause. There is, then, apparently no relationship between the ionic permeability properties of the membrane and water permeability.

A relationship between rate of water loss and metabolic activity has often been observed (see Hill, 1908; Hall, 1922; Ludwig, 1936). Ludwig's (1936) evidence is based on a more rapid loss of water during initial periods of desiccation, when the larvae are active. Later the larvae become inactive, and rate of water loss is less.

Further, in the pupal stages, desiccation is slowest. Respiratory rates are also low at this stage, as Ludwig (1931) has already shown. In the present study on the grasshopper egg, the same slow rate of desiccation was found for the diapause stage. The rate of desiccation apparently seems related to the general metabolic activity; but the situation, however, is not quite so simple. In isolated grasshopper embryos the rate of shrinking in 5 N Belar is as rapid for diapause as for postdiapause animals. In centrifuged eggs, internal organization is disrupted; yet the same qualitative difference in desiccation rate is evidenced as for non-centrifuged diapause and postdiapause eggs. Finally, diapause and postdiapause eggs lose water as rapidly in carbon monoxide–oxygen mixtures as in air. Such gas mixtures depress the respiratory rate of the postdiapause group but have little or no effect on the respiration of diapause eggs.


## SUMMARY

1. The rate of water loss of *Melanoplus differentialis* eggs at different developmental stages has been determined. No qualitative difference in respect to rate was found between wet (with hypertonic solutions) and dry ($CaCl_2$) dehydration. The resistance to desiccation decreases with morphological age of developing eggs. Diapause eggs are most resistant.

2. The rate of desiccation does not seem to be closely associated with metabolic activity, as represented by that fraction of the respiration which is dependent on structure, or which may be depressed by certain $CO/O_2$ mixtures.

3. The oxygen consumption of dehydrated eggs decreases during water loss. In no case was an increase observed during dehydration.

4. The vital limit of desiccation of postdiapause eggs at 25° C. was found to be about 46 per cent of the initial water.


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# VARIATIONS IN ACIDITY AND OXIDATION-REDUCTION POTENTIAL OF RODENT UTERINE FLUIDS[1, 2]

(Five figures)

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MANY morphological and physiological studies of the rodent's uterus have been made in the course of research into problems of embryology, reproduction, and endocrinology; yet little information concerning the fluids contained within the uterine lumen is available. This is surprising since the uterine fluids, affording the immediate environment for both egg and sperm within the uterine cavity, may affect directly the viability and implantation of the blastocysts as well as the viability and motility of the spermatozoa.

This investigation was undertaken to determine the hydrogen-ion concentration and oxidation-reduction potential of the uterine fluids *in situ* in the rat and mouse during the normal oestrus cycle, pseudopregnancy, and early pregnancy. These two characteristics of the uterine fluid were chosen for study, first because studies *in vitro* have demonstrated that the H-ion concentration and, to a lesser extent, the oxidation-reduction potential of media greatly influence the activity and longevity of spermatozoa and the development of eggs; and secondly because they can be determined quantitatively in living animals.

The literature describing the nature and origin of the uterine fluids in rodents is largely the record of observations made incidental

[1] Revision of a dissertation presented to the faculty of the Graduate School, Yale University, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[2] I wish to express my gratitude to Dr. J. S. Nicholas, under whose direction this investigation was begun and completed, for helpful advice and criticism and especially for his constant and kindly interest. I also wish to thank Dr. T. L. Jahn for valuable technical advice and for the use of his vacuum-tube potentiometer and other apparatus.

to histological studies of the uterus. An exception to this is the paper of Bond (1898), who investigated the secretory function of the uterus in the guinea pig and the rabbit and described the fluid collecting in a ligated uterine horn as a colorless, watery fluid, neutral or faintly alkaline in reaction, of very low specific gravity, containing some albumin and mucin and a remarkably large proportion of sodium chloride.

Emrys-Roberts (1905), and later Stockard and Papanicolaou (1917), described in the guinea pig abundant secretion of mucus by uterine epithelial cells during pro-oestrum.

Long and Evans (1922) and E. Allen, (1922), in their respective monographs on the oestrus cycles of the rat and mouse, state that toward the latter part of pro-oestrum the uterine cornua frequently become distended with a clear, watery, non-coagulable fluid, elaborated by the uterine glands.

The uterine fluids may be also partly of tubal and ovarian origin; but of the numerous papers dealing with the cyclic functional and structural changes in tubal epithelium, reference will be made only to the excellent review of Novak and Everett (1928), who were able to demonstrate changes in the human tube correlated with cyclic ovarian changes.

A number of histological and cytological studies of uterine mucosa in rats and mice, which have been made with modern staining techniques, reveal cyclic variations in the staining reaction of the uterine epithelium (Eisler, 1926; Glas, 1930; Reese, 1931).

The only recent investigation concerned chiefly with the nature of the uterine fluid is that of Aasland (1932), who attempted to lower the H-ion concentration of the uterine secretion in rabbits by producing a condition of acidosis in test animals. The pH determinations were made electrometrically with antimony electrodes inserted into the cervical canals of anesthetized rabbits. Aasland found that the pH of the cervical secretion of the experimental animals which did not differ significantly from that of the control animals varied from pH 7.85 to 8.40.

In an extensive investigation of the biology and physiology of spermatozoa and of vaginal secretions in cattle, Roemmele (1927), using a litmus-paper indicator, found that the H-ion concentration

of the vaginal fluids varied from pH 7.2–7.5 to pH 7.8–8.5. Roemmele was unable to establish a definite difference in the H-ion concentration of oestral, sterile, or pregnancy mucus.

The cyclic anatomical changes of the uterus in the rat and mouse during the normal oestrus cycle, pseudopregnancy, and early pregnancy have been carefully studied and correlated with the functional state of the ovary by Long and Evans (1922), E. Allen (1922), Parkes (1926, 1929), W. M. Allen (1931), and Clauberg (1931). These excellent studies have been used by the author in establishing the morphological condition of the uterus in respect to the various stages of the sexual cycle as determined by the vaginal-smear method.

## MATERIALS AND METHODS

*Animals.*—The animals, mature female mice and rats, were studied under sodium amytal anesthesia. The rats were given the regular female dosage of 0.01 cc. of a 10 per cent aqueous solution of sodium amytal per 10 gm. of rat (Nicholas and Barron, 1932); but the dosage had to be slightly increased to produce complete anesthesia in those animals which were injected daily, as a tolerance for the amytal was acquired.

After experimenting on over a hundred animals, males and females, a dosage of 0.005 cc. of 4 per cent sodium amytal per gram of mouse was adopted as most satisfactory. Individual mice may react very differently to sodium amytal. A sufficient dosage for complete anesthesia in one animal may be inadequate in another, and lethal to a third. The decided difference in the reaction of males and females to sodium amytal, as found by Nicholas and Barron for the rat, does not hold for mice. In the mouse, sodium amytal is equally efficacious in males and females.

*Apparatus.*—The pH measurements were made by means of glass electrodes of special design which could be inserted through the vagina and os uteri into the uterine horn of the anesthetized animal without causing appreciable injury to the uterus or cervix. The glass electrodes were constructed from glass (Corning glass No. 015) of the composition recommended by MacInnes and Dole (1930). Two designs of electrodes were employed, a modification of the MacInnes and Dole type (see also Du Bois, 1932) and a capillary

type (Fig. 1*a*) similar to that described by Voegtlin and Kahler (1932). Electrodes of the MacInnes and Dole type were easily broken, and their performance when used in the uterus was less satisfactory than that of the capillary-tube type electrodes described below. They were discarded in favor of the capillary-tube electrode.

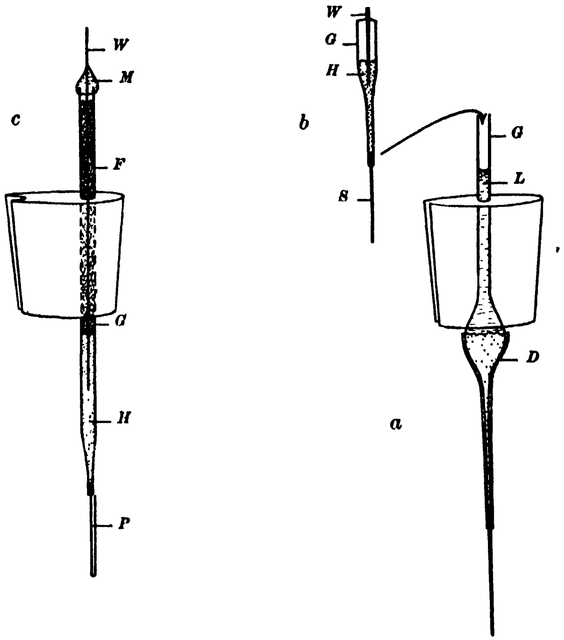


FIG. 1*a*, *b*, *c*.—Diagrams of electrodes used in making intra-uterine measurements: *a*, glass electrode; *b*, Ag/AgCl reference electrode; *c*, platinum electrode; *W*, copper wire; *M*, seal; *F*, cotton; *G*, glass tube; *H*, mercury; *P*, platinum; *S*, silver-coated platinum wire; *L*, M/10 HCl.

Capillary-tube electrodes which functioned satisfactorily electrically were too fragile for practical use in the uterus when they were made by drawing the capillary directly from glass tubing 5 mm. in diameter. To offset this difficulty a thin bulb, 15–20 mm. in diameter, was blown in a short section of the tubing, and the capillary was drawn from the bulb. The walls of the capillaries were not of uniform thickness but varied from 10 to 16 $\mu$ in the better electrodes.

The deviation film (see Kahler and De Eds, 1931) was eliminated by coating the bulb and capillary portion of these electrodes with

De Khotinsky cement. The electrodes were filled with M/10 HCl. Air bubbles were removed by suction and heating in a current of hot air. The electrodes were fitted into niched corks and kept in vials with the tips of the electrodes immersed in distilled water. The glass electrodes were then equipped with Ag/AgCl platinum-wire electrodes (Fig. 1*b*) and their electrode function tested. All glass electrodes showing high unstable asymmetry potentials were discarded.

The electron-tube potentiometer used in these experiments was built by Dr. T. L. Jahn (Jahn, 1935). The vacuum-tube potentiometer was built into a portable unit separate from the constant-temperature oven which housed the electrodes. Since the constant-temperature oven was copper-lined, all electrodes, animals, etc., were placed upon a false bottom, formed by a glass plate resting on paraffined corks. A Leeds and Northrup Type 2500*e* galvanometer was used in connection with a Type K potentiometer. Potentiometer readings were read to the nearest millivolt.

The platinum electrodes used to obtain the oxidation-reduction potentials were made from No. 26 platinum wire fused into a glass tube (Fig. 1*c*). Mercury was used in making electrical connection with the platinum wire. The best results were obtained with the electrode wire bent back on itself. This design both increased the active electrode surface and left the anterior end blunt, so that the delicate uterine tissues were less readily injured.

A silver-silver chloride electrode similar to the microelectrode described by Taylor (1925) was used as a leading-off and reference electrode. The Ag/AgCl reference electrode and the glass electrodes, when in use, were supported by individual stands (see Fig. 2). These stands had high insulating properties, were convenient to use, and were simply made by bending glass rods of appropriate lengths into the desired shape. The long end of the glass rod was fitted into a cork attached with sealing wax to a 6 cm. square of heavy plate glass to give the stand stability. The niched cork holding the leading-off electrode was attached to the supporting arm of such a stand by means of a rubber band.

*Technique.*—To hold the animal in position so that the ora uteri could be observed to facilitate the insertion of electrodes, a simple

stand was used (Fig. 2). The anesthetized animal was fastened to the stand by its tail, which was held to the sloping surface of the stand with adhesive tape. By holding the vagina expanded with a

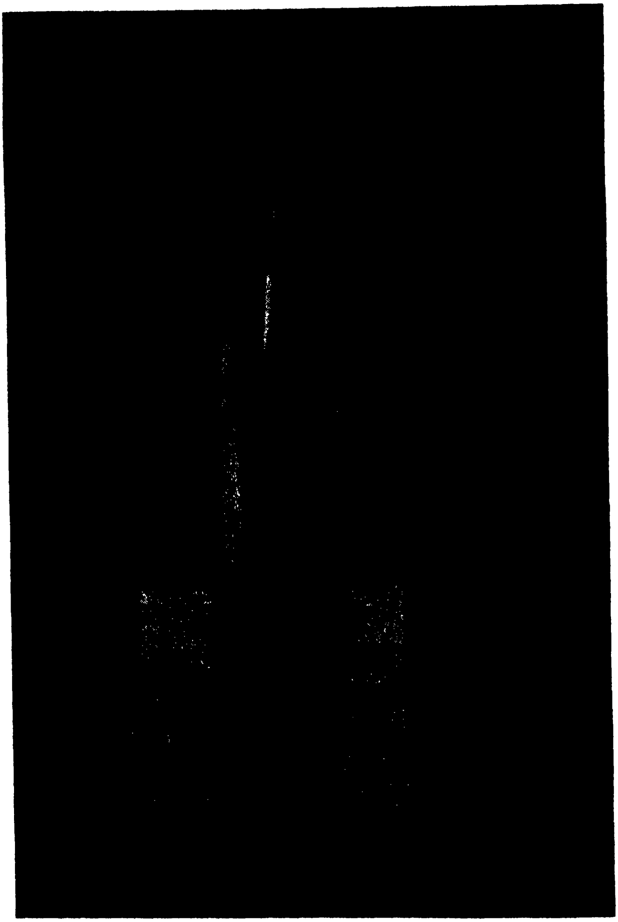

FIG. 2.—Anesthetized animal with glass electrode and reference electrode in position for taking pH readings.

small spring-brass speculum and a pair of curved forceps, and with a Nicholas lamp focused from above into the vaginal orifice, it was usually possible to recognize the ora of the right and left uterine horns as the electrode was inserted. The inserted electrode was held

in position by placing a rubber band around the supporting rod attached to the stand and the niched cork holding the electrode.

Before the electrodes were inserted, a vaginal smear was taken. The smears were fixed in ether-alcohol solution and stained with Erlich's haematoxylin, eosin, and water blue.

In making pH and Eh measurements the following routine was followed: The glass electrode was placed in the left uterine horn of an animal, and readings were taken for 10–15 minutes. The electrode was then removed, washed in distilled water, and inserted into the right horn. At times, especially if difficulty was met in entering the os of one side, determinations were made in one horn only. Oxidation-reduction potentials were taken in one cornu only, as it frequently took 30–60 minutes or more for the platinum-wire electrode to reach an equilibrium. The platinum electrodes were cleaned in chromic sulphuric acid cleaning mixture and were well washed in running water before use.

After an electrode had been inserted into the uterine horn, the animal was placed in the constant-temperature oven and the lead-off-reference electrode was placed on the animal. The practice that gave the most consistent and reliable results was to insert a piece of filter paper into the rectum of the animal, leaving the end of the paper projecting a few millimeters from the anus. The filter paper was then drenched with M/10 KCl until a drop of the KCl solution would remain on the paper. The tip of the reference electrode was placed in this drop of KCl solution. The potentiometer leads were then clipped to the electrodes and readings taken.

It was found advisable, and adopted as routine, in making all pH measurements in the uterus to check the observation before and after each determination by measuring the e.m.f. produced by the glass electrode in two standard buffer mixtures, one slightly more and one slightly less acid than the uterine pH. Using this method, the required pH can be interpolated, and errors due to the performance of the electrode are largely eliminated.

The drift and fluctuation in the potential developed by the glass electrodes in the uterine lumen was considerable at times, but the average drift was not over 6 mv. Although the pH values listed in the tables are calculated from readings which include the drift, the

average error probably does not exceed 0.05 pH. Voegtlin and Kahler (1932), who first used the capillary electrode glass in estimation of the H-ion concentration of tissues in living animals, found similar drifts; and they believe that with sufficient repetition of the measurements the average error of the method is not greater than 0.05 pH.

The MacInnes and Dole type of microelectrodes usually developed potentials indicating H-ion concentrations 0.1–0.3 pH less than a capillary-tube type of electrode inserted into the same uterine horn. This tendency is reflected in the low pH averages of Mouse Series II as compared to those of Mouse Series III.

The oxidation-reduction potential is established by a complex equilibrium of all the ions characterizing the system measured, and is influenced by the H-ion equilibrium as well as the specific oxidation-reduction equilibria. The oxidation-reduction potentials drifted from high positive values when first measured to more negative values as equilibrium was neared. This drift has been found by others in similar experiments with platinum electrodes, and is usually considered to be due to the presence of oxygen carried in on the electrode. Because fluctuations of 20–30 mv. in the platinum electrode potentials often occurred within a few minutes of each other, it was difficult to decide when an equilibrium had been reached. For this reason the final oxidation-reduction values listed in the tables may, in individual cases, be in error as much as 50 mv.

The standard buffer mixtures were Sørensen's phosphate buffer (Clark, 1928). The M/15 $Na_2HPO_4$ and M/15 $KH_2PO_4$ stock solutions were stored in well-stoppered 2-liter flasks equipped with soda-lime tubes, and the desired buffers were titrated fresh daily. The pH values of the phosphate buffer mixtures were checked to 0.03 pH by means of the quinhydrone electrode against standard M/10 HCl.

In classifying and referring vaginal smears to the various oestral stages, the following system of notation and classification has been adapted from Long and Evans (1922) and Allen (1922). A brief, generalized summary of the structural changes characterizing the uterus is included:[3]

[3] The letters "e" and "l" are occasionally used in connection with "I" and "II" to indicate that the smear has elements characteristic of the stage preceding or following it.

I.—Pro-oestrus smear: only nucleated epithelial cells. Uterine epithelium columnar and intact; marked distention of lumen and glands; leucocytes in stroma.

II.—Oestrus smear: only cornified cells not in large clumps or masses. Uterine epithelium cuboidal; degeneration begins in late II; glands and stroma much as in I.

III (e).—Early metoestrus smear: large clumped masses of cornified cells. Uterine epithelium undergoes vacuolar degeneration.

III (l).—Late metoestrus smear: cornified elements present, also leucocytes and nucleated epithelial cells. Uterine epithelium largely degenerate; no basement membrane, but repair of epithelium begins; glands show a minimum of function.

IV.—Dioestrus smear: leucocytes and usually stringy mucus with nucleated epithelial cells. Smear IV is found during the dioestrus interval of the normal cycle and throughout the period of pregnancy and pseudopregnancy.

## EXPERIMENTAL SECTION

*Experimental Series I.*—Preliminary attempts to measure the H-ion concentration of uterine fluids colorimetrically were unsatisfactory and led to the adoption of the electrometric method. In these experiments the uterine horns were carefully excised and slipped over the electrodes. The uterine horn with the inserted electrode tip was then placed in a bath of Ringer's solution in the constant-temperature oven. Connection between the Ringer's solution and a beaker of M/10 KCl into which dipped the reference electrode was made by an agar-KCl bridge. Solution temperatures within the oven varied from 36° to 40° during the course of an experiment. pH readings were taken every 2 or 3 minutes for 5–60 minutes; then the glass electrode was replaced by a platinum electrode and Eh readings were obtained. Both horns of each uterus were similarly treated, but usually a delay of at least 10 minutes intervened between the initial pH readings of the right and left horns.

The results of these experiments, ten of which are tabulated in Table I, indicated that pH and Eh measurements of the fluids in the lumen of excised uteri are not reliable criteria of these characteristics in the living animal, for the initial pH and Eh readings were always followed by drifting electrode potentials. As the readings were continued in excised uteri, pH values became increasingly acid and the Eh values more negative.

To test the behavior of the glass electrode in the uterine lumen of a living animal, a mouse was anesthetized and the left uterine horn was exposed via a median ventral incision. A glass electrode was inserted into the lumen of the horn through an incision near the tubal-uterine junction. The animal was placed in the constant-temperature oven, and connection was established with the reference electrode by placing an agar-KCl bridge on the base of the uterus and

TABLE I

THE PH AND EH VALUES OF FLUIDS IN LUMEN OF EXCISED MOUSE UTERI*

| Mouse | Oestrus Stage | Horn | pH Values Initial-Final | | Eh in mvs. | |
|---|---|---|---|---|---|---|
| A9M1 | I | rt.<br>lft. | 6.87 – .37<br>6.30 – .31 | (35)<br>(30) | +169<br>+148 | (20)<br>(90) |
| A5M1 | II | rt.<br>lft. | 6.23<br>6.23 – .17 | (0)<br>(20) | +164 | (20) |
| A13M2 | II | rt.<br>lft. | 7.48 – 6.78<br>6.75 – .27 | (15)<br>(38) | +111<br>+198 | (30)<br>(30) |
| A16M1 | II | rt.<br>lft. | 6.90 – .40<br>6.88 – .40 | (60)<br>(45) | +193<br>+250 | (60)<br>(60) |
| A5M2 | III (1) | rt.<br>lft. | 6.39 – .30<br>6.58 – .57 | (5)<br>(13) | +111 | (75) |
| A11M1 | IV | rt.<br>lft. | 7.00 – .52<br>6.40 – .45 | (15)<br>(20) | +195 | (60) |
| A13M1 | IV | rt.<br>lft. | 6.88 – .43<br>6.47 – .31 | (20)<br>(25) | +240<br>+242 | (60)<br>(50) |
| A13M3 | IV | rt.<br>lft. | 6.83 – .37<br>6.65 – .37 | (34)<br>(15) | +179<br>+183 | (15)<br>(15) |
| A13M4 | IV | rt.<br>lft. | 6.93 – .40<br>6.40 – .22 | (30)<br>(30) | +95<br>+88 | (21)<br>(30) |
| A11M2 | Preg. 50–60 Hrs. | rt.<br>lft. | 6.80 – .52<br>6.67 – .52 | *(25)<br>*(15) | +59 | *(120) |

* Numbers in parentheses represent time elapsed in minutes between initial and final readings.

moistening the uterine-bridge junction with M/10 KCl. The pH values obtained during 25 minutes' observation fluctuated between pH 7.13 and 7.40 but showed no tendency to fall to the acid values found in the excised uteri. The opposite horn was excised and, using the same electrode, pH readings were taken as before. The initial value was pH 6.45, and this soon dropped to pH 6.32. This experiment demonstrates that the H-ion concentration of uterine fluids in excised uteri is different from that which obtains in the living ani-

mal; so the following series of experiments were undertaken to investigate the pH and Eh of the fluids in the uteri of living animals.

*Experimental Series II.*—In this and the following series of experiments all observations were made on living animals. The pH and Eh measurements were made by inserting the electrodes into the uterine horns via the vaginal canal. The data obtained in the study of thirty mice are listed in Table II. The pH values found in this and the following tables under the heading "Initial" are not in all cases calculated from the first potentials obtained after the insertion of electrodes but at times from potentials recorded 3–5 minutes later. These values were used when the initial potentials fluctuated so widely as to appear unreliable.

The most alkaline values of the uterine fluids were obtained in animals in metoestrum, while the lowest pH values were found generally in uteri of animals in pro-oestrum. The low pH recorded for one horn of animal A30M6 in metoestrum is an exceptional case, perhaps the result of error in obtaining the pH. The pH of the uterine fluids of the animals in oestrus varied from a value similar to the average pro-oestrus pH to values as high as those obtained in the metoestrus group. The pH obtained in the uteri of the diosetrus animals averaged lower than that of the metoestrus animals and higher than that of the other groups. There were no very low or very high values in this group. The average pH value obtained in the pregnant animals was lower than that recorded for the dioestrus mice.

To test whether the means listed in the foregoing and following sections differ significantly, the data were treated by the method of Fisher (1930) for testing the significance of difference of means of small samples. A value of 0.05 or less for $P$ has been considered as indicative of a significant difference of two means. By this standard, the pH means of Table II are significantly different for the pro-oestrus and oestrus group ($P=0.04$), for the dioestrus and pro-oestrus group ($P=0.01$), and for the oestrus and metoestrus group ($P=0.01$); but the differences of the pH means of the metoestrus and dioestrus groups and of the dioestrus and pregnant animals are of less significance ($P=0.14$ and $0.2$).

The Eh means of all the groups, excepting the metoestrus group,

TABLE II

THE pH AND EH VALUES OF UTERINE FLUIDS *in situ* AS FOUND IN 30 LIVING MICE*

| Mouse | Oestrus Stage | pH Values Initial-Final | Drift in pH | Av. pH | Eh in mv. |
|---|---|---|---|---|---|
| A18M2 | I | 7.08 - 7.05 (55) | 0.03 | 7.07 | +185 (60) |
| A21M1 | I | 6.88 - 6.96 (15) | 0.08 | 6.92 | +195 (40) |
| A21M2 | I | 6.89 - 7.09 (30) | 0.20 | 6.99 | +146 (30) |
| A22M2 | I | 7.00 - 7.00 (15) | 0.00 | 7.00 | +184 (10) |
| A22M4 | I | 6.90 - 6.77 (10) | 0.13 | 6.84 | +289 (40) |
| A31M2 | I | 6.97 - 6.97 (25) | 0.00 | 6.97 | +233 (40) |
| For pH mean, $\sigma$=0.09 Average= | | | 0.07 | 6.97 | +205, $\sigma$= 46 |
| A22M1 | II | 7.05 - 7.05 (10) | 0.00 | 7.05 | +210 (10) |
| A23M2 | II | 6.85 - 7.00 (20) | 0.15 | 6.93 | +165 (60) |
| A23M3 | II | 7.17 - 7.40 (40) | 0.23 | 7.29 | |
| A30M2 | II | 7.10 - 6.98 (10) | 0.12 | 7.04 | +222 (35) |
| A31M3 | II | 7.20 - 7.10 | 0.10 | 7.15 | +205 |
| A31M1 | I (l) | 7.13 - 7.20 (35) | 0.07 | 7.17 | +233 (40) |
| A30M8 | I (l) | 7.11 - 7.14 (15) | 0.03 | 7.13 | +228 (45) |
| For pH mean, $\sigma$=0.115 Average= | | | 0.10 | 7.11 | +211, $\sigma$= 25 |
| A29M1 | III (e) | 7.20 - 7.35 (40) | 0.15 | 7.28 | |
| A30M3 | III (l) | 7.40 - 7.33 (45) | 0.07 | 7.37 | +185 (70) |
| A30M6° | III rt.<br>(e) lft. | 6.75 - 6.86<br>7.25 - 7.23 (60) | 0.11<br>0.02 | 6.81<br>7.24 | +139 (10) |
| A30M7 | III (l) | 7.57 - 7.43 (45) | 0.14 | 7.50 | +150 (77) |
| For pH mean, $\sigma$= 0.115 Average = | | | 0.10 | 7.35 | +158, $\sigma$=24 |
| A18M1 | IV | 7.05 - 7.08 (20) | 0.03 | 7.07 | +205 (20) |
| A22M3 | IV | 7.23 - 7.30 (30) | 0.07 | 7.27 | +205 (30) |
| A23M1 | IV | 7.12 - 7.20 (60) | 0.08 | 7.16 | |
| A29M2 | IV | 7.30 - 7.38 (20) | 0.08 | 7.34 | |
| A29M5 | IV | 7.37 - 7.34 (20) | 0.03 | 7.36 | +241 (45) |
| A30M4 | IV | 6.95 - 6.90 (33) | 0.05 | 6.93 | +221 (25) |
| A31M4 | IV | 7.27 - 7.30 (15) | 0.03 | 7.29 | +153 (75) |
| For pH mean, $\sigma$=0.158 Average = | | | 0.05 | 7.20 | +205, $\sigma$= 33 |
| Preg. Mice | | | | | |
| A18M3 | 50-60 hr. | 6.97 (30) | | 6.97 | +206 (90) |
| A25M1 | 60 hr. | 7.05 - 7.25 (20) | 0.20 | 7.15 | +256 (20) |
| A25M2 | 30 hr | 7.07 - 7.13 (22) | 0.06 | 7.10 | +202 (33) |
| A29M3 | 50-60 hr. | 7.05 - 7.13 (30) | 0.08 | 7.09 | +198 (45) |
| A20M1 | 40 hr. | 7.10 | | 7.10 | |
| B1M2 | 72 hr. | 7.20 - 7.13 (30) | 0.07 | 7.17 | +209 (25) |
| For pH mean, $\sigma$ = 0.083 Average= | | | 0.10 | 7.10 | +214, $\sigma$= 24. |

* Numbers in parentheses indicate duration of readings in minutes

° pH average does not include value of rt. horn of A30M6.

$$\sigma = \sqrt{\frac{\Sigma(X-\bar{X})^2}{n-1}}$$

vary from +201 to +214 mv. and do not differ significantly. Only three Eh readings were obtained from animals showing metoestrus smears; but all three values are low, and the average (+158 mv.) differs significantly from the other means.

*Experimental Series III.*—H-ion determinations of the uterine fluids *in situ* were made daily for 8–17 days on eight mice. Oxidation-reduction potentials were also obtained, but not daily. The pH observations are summarized Figure 3, in Graphs I-VIII, and the Eh measurements in Table III. Because the glass electrodes did not maintain a definite equilibrium potential within the uterus, the pH values used in preparing the graphs were calculated from the average of two readings, the first taken soon after the insertion of the electrode, the other taken 10–15 minutes later. The average fluctuation corresponds to a deviation of less than ±0.04 pH from the mean value used in plotting the graphs.

The limits of variation of the observed pH of the normal uterine fluids in these animals were pH 6.93 and pH 7.70. The pH curves show decided fluctuations, which in general appear to be cyclic and correlated with the onset, culmination, and disappearance of cornified cells in the vagina. Minimum alkalinity in the uterine fluid was generally associated with pro-oestrum (average pH 7.09±0.13). This was followed by increasing alkalinity of the uterine fluid through oestrus (average pH 7.27±0.15), with the maximum alkalinity generally coinciding with the late cornified stages of the vaginal smear. The average maximum alkalinity was pH 7.43±0.13. These mean values differ significantly from each other.

The mean value correlated with all the early metoestrus smears was pH 7.24; but if the observations made during the periods of abnormal vaginal cornification in Mouse III and VI are omitted, the mean becomes the significant value, pH 7.35±0.17. The latter is probably the more accurate indication of the H-ion concentration of the fluid within a typical metoestrus uterus. A decreased alkalinity in the uterine fluid generally accompanied the advent of leucocytes into the vaginal smear. The pH curves during late metoestrum and dioestrum fluctuated erratically; but the average pH measured in late metoestrus uteri was similar to that recorded for dioestrus uteri, both being pH 7.24. The erratic fluctuations in the uterine pH

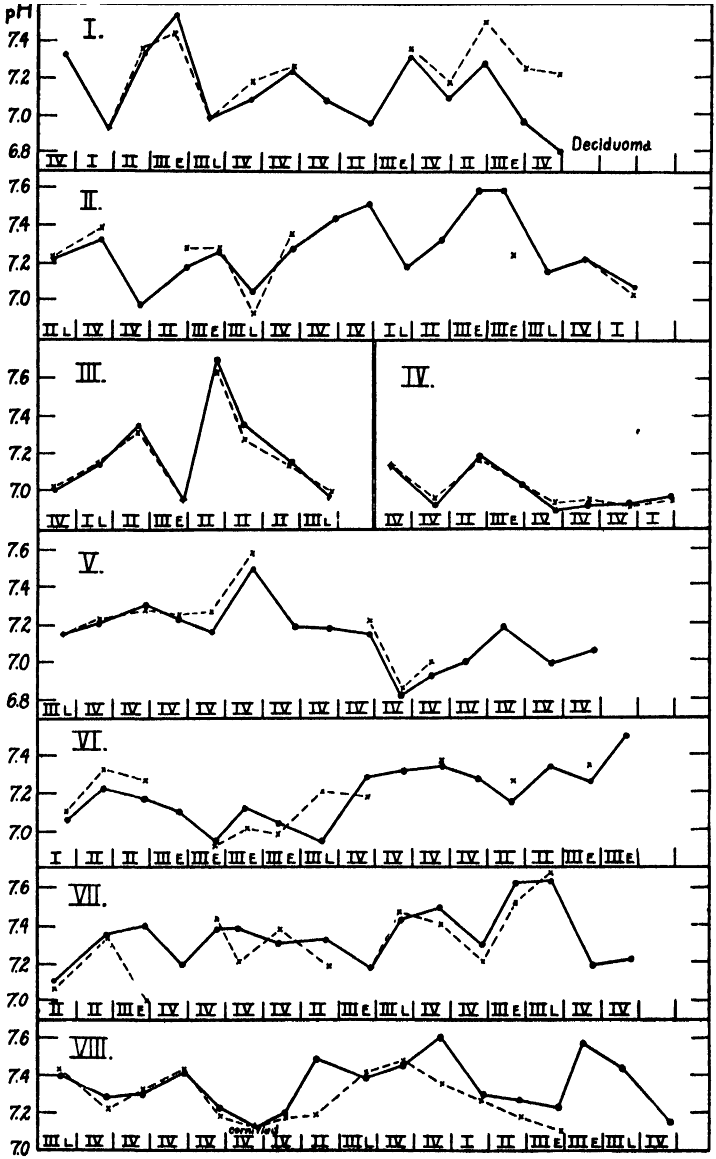


FIG. 3.—The H-ion concentration of the uterine contents of eight mice studied daily for 8–16 days. Vertical axis, pH values; horizontal axis, time in 24-hour intervals and type of vaginal smear at time of pH observation. Solid lines indicate left cornua; broken lines, right.

and abnormal vaginal smears observed in some of these animals may be due in a large measure to physiological disturbances arising from the daily anesthetization and insertion of electrodes.

Attention is drawn to the unexpected situation which developed in Mouse I. The vaginal-smear record indicated that three oestrus cycles occurred during the observations: yet on the fourteenth day of observation inspection of the uterus revealed the presence of a large deciduoma in the left cornu.

In relation to the results of the experiments on excised uteri, it is significant that the pH obtained in the uterus of Mouse VI, 2 minutes after its death, was the acid value pH 6.65. This value is nearly ten times as acid as that observed in the same animal 16 hours before.

TABLE III

OXIDATION-REDUCTION POTENTIALS OBTAINED IN UTERINE HORNS OF MICE OF SERIES III

| Oestrus Stage | I | II | IIIE | IIIL | IV |
|---|---|---|---|---|---|
| Eh | +275 | +263 | +165 | +240 | +215 |
| " | +198 | +141 | | + 93 | +234 |
| " | + 83 | | | +192 | +208 |
| " | | | | | +282 |
| " | | | | | +240 |
| " | | | | | +265 |
| " | | | | | +259 |
| " | | | | | +222 |
| Average Eh | +185 | +202 | +165 | +175 | +241 |
| $\sigma$ | 97 | 86 | | 75 | 26 |

The oxidation-reduction potentials listed in Table III are similar to those found in the preceding experiments.

*Experimental Series IV.*—These experiments consist of daily pH and intermittent Eh observations made upon eight rats during a period of 16–17 days. The results of these observations are summarized in Figures 4 and 5, Graphs IX–XVI, and Table IV. The potentials developed by the glass electrodes within the rat uteri fluctuated relatively little. Although occasionally there were large

drifts up to 25 mv., the average was about 4 mv., which corresponds to a deviation of $\pm 0.03$ pH from the mean value used in plotting the graphs.

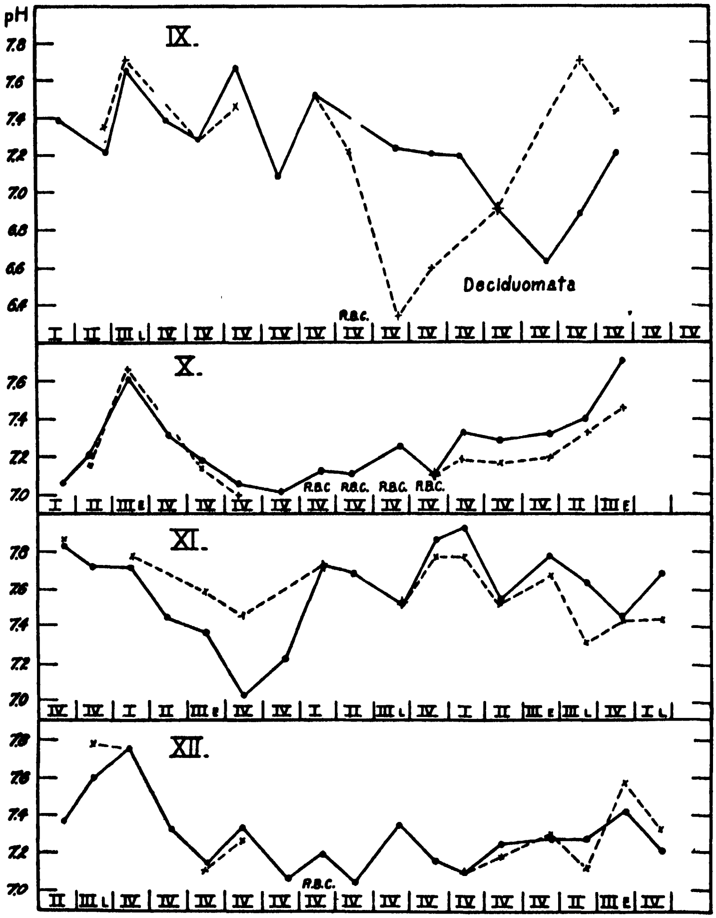


FIG. 4.—The H-ion concentration of the uterine contents of rats 1–4 studied daily for 17 days. *R.B.C.* indicates erythrocytes were present in vaginal smears; otherwise figure explanation the same as for Figure 3.

The H-ion concentration of the normal uterine fluids of the eight rats varied from pH 7.93 to 6.95. As in the mice the uterine fluids in the rats were characterized by a more acid nature at the onset of

oestrus than the fluids of the late oestrus or early metoestrus uterus. The record of Rat 2 closely approximates the average of all observations in this series. The results obtained in every animal are similar

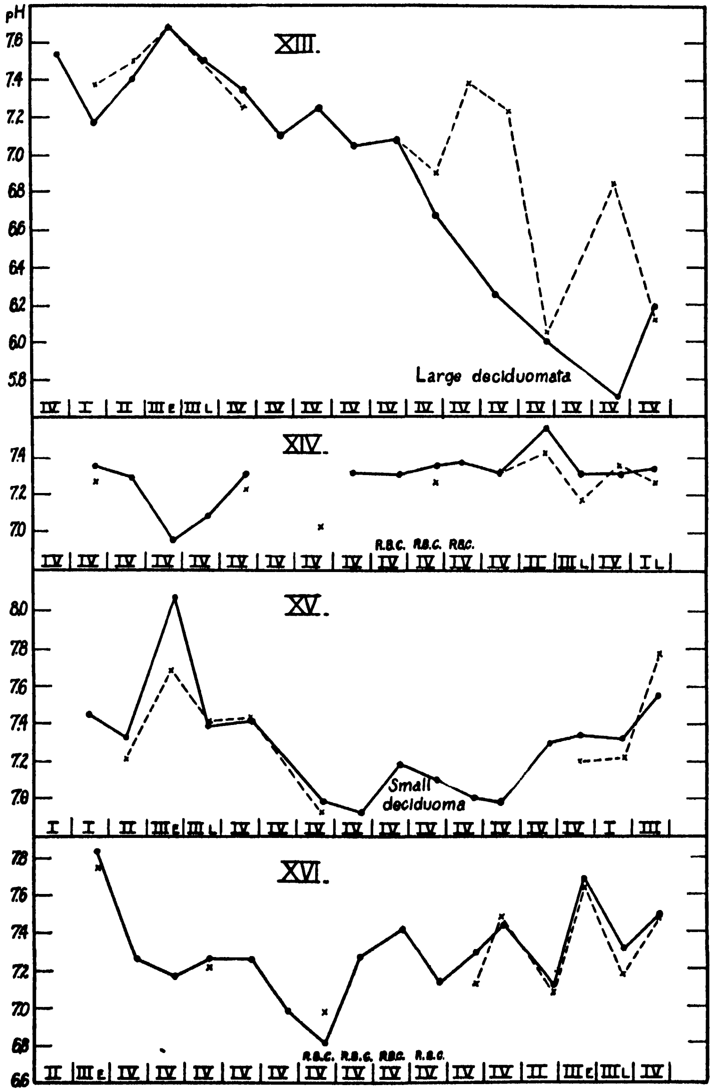


FIG. 5.—The H-ion concentration of the uterine contents of rats 7–10 studied daily for 17 days. Figure explanation the same as for Figures 3 and 4.

and consistent, except for the record of Rat 3, which will be considered separately since it offers several peculiarities.

As in the mouse series, the pH minimum preceding the increase in alkalinity of the uterine fluid was associated with either a stage IV, I, or at times a II smear. The average pH obtained in pro-oestrus uterus was $7.29 \pm 0.14$, and in the oestrus uterus $7.31 \pm 0.14$. Readings taken 24 hours after oestrus readings, whether the vaginal smear was stage III (e) or III (l), showed a peak value in the alkaline direction.

The average alkalinity during stage III (e) was $7.69 \pm 0.08$; and during stage III (l), pH $7.43 \pm 0.20$. After the maximum alkalinity was attained, the uterine fluid rapidly became less alkaline, so that the average pH recorded during the first three days of stage IV was pH $7.26 \pm 0.18$. The average increase in acidity during this period was 0.5 pH. The foregoing data show that there is a marked significant increase in the alkalinity of the uterine fluid associated with the phenomena of oestrus, which is followed by an increased acidity in the following dioestrum.

The smear records show that six, and possibly seven, of the eight animals became pseudopregnant soon after the observations were begun. Although Rat 7 yielded a dioestrus smear for 13 days, it is possible that it was merely going through an extended interval period and not a period of pseudopregnancy. The pH obtained in the uterine lumen during the critical period 4–6 days after oestrus in the six pseudopregnant animals varied from 6.90 to 7.50, the average value being the significantly low pH $7.14 \pm 0.17$. A general average of the pH values obtained during the latter part of the pseudopregnancy cycle has little meaning in view of the widely different conditions occurring in the separate horns during this period.

Acid values from pH 6.80 to 5.72 were always associated with the presence of deciduomata in the uteri. The induction of the deciduomata was the result of accidental injury to the uterine mucosa during the "sensitive period" occurring 4–6 days after the induction of the condition of pseudopregnancy. Since the two uterine horns of an animal were not always investigated and injury was a matter of chance, it was possible for one cornu to develop deciduomata independently of the other cornu. This accounts for the great difference

between the pH curves of the two uterine horns of some animals. The large and erratic fluctuation in the pH curve of the right horn of Rat 6 on the sixteenth day of observation may have been caused by contamination of the electrode by blood and cellular débris which was present in the vagina. Small deciduomata were found in the anterior halves of both uterine horns of Rat 9 on the twelfth day of observation. Four days later the animal began a new oestrus cycle, and the alkalinity of the uterine lumen increased 0.6 pH.

The mean Eh values (Table IV) occurring in dioestrum and during the 4–6 and 7–14 days of pseudopregnancy differ significantly

TABLE IV

OXIDATION-REDUCTION POTENTIALS OBTAINED IN UTERINE HORNS OF RATS OF SERIES IV

| | Stage of Oestrus Cycle | | | | | Pseudopregnant | | Deciduoma |
|---|---|---|---|---|---|---|---|---|
| Eh | I | II | III (e) | III (l) | IV | 4 to 6 | 7 to 14 Days | |
| " | +257 | +70 | | +248 | +276 | +265 | + 253 | + 173 |
| " | | +276 | | +195 | +257 | +267 | + 232 | + 176 |
| " | | | | | +280 | +356 | + 296 | + 134 |
| " | | | | | +275 | +288 | + 291 | |
| " | | | | | +245 | +295 | + 258 | |
| " | | | | | +190 | +315 | + 278 | |
| " | | | | | +160 | +308 | + 233 | |
| " | | | | | | +232 | + 245 | |
| " | | | | | | | + 210 | |
| Average Eh | +257 | +173 | | +222 | +240 | +291 | + 255 | + 161 |
| $\sigma$ | | | | | 47.2 | 31.3 | 28.8 | 23.4 |

($P = < 0.01$). The oxidation-reduction potentials obtained in the deciduomatous tissue average to the significantly low value of +161 mv. The values obtained during the fourth to sixth day of pseudopregnancy are all above +232 mv., and the values obtained during the latter half of pseudopregnancy are all above +210 mv.

Rat 3 was the only animal in this series which did not present a continuous dioestrus smear soon after the experiment began, and the pH curves of this animal are not consistent with those obtained in the other seven. Using the pH average for the other seven animals as a standard, the pH curve for this animal is abnormally high during stages IV and I. The average pH value during stage IV was pH

7.50 and during stage I pH 7.71, as compared to pH 7.26 and pH 7.29 for the other animals in this series. That this animal presents an atypical case is further supported by breeding experiments, which proved the animal to be sterile.

*Experimental Series V.*—The data of the pH and Eh observations made upon four rats in early pregnancy are summarized in Table V. In all cases the tubes were removed from these animals either before or directly after the observations listed here were made, and developing eggs were found in the two- and four-celled stages. The pH and

TABLE V

THE pH AND Eh VALUES OF UTERINE FLUIDS *in situ* IN RATS DURING EARLY PREGNANCY

| Rat | Horn | pH Values Initial-Final | Drift in pH | Av. pH | Eh in mv. | Remarks. |
|---|---|---|---|---|---|---|
| B13R1 | lft.<br>rt. | 7.46 - 7.49<br>7.50 - 7.54 | 0.03<br>0.04 | 7.48<br>7.52 | +255 | Preg. 48 hrs. |
| B13R2 | lft.<br>rt. | 7.42 - 7.40<br>7.44 - 7.42 | 0.02<br>0.02 | 7.41<br>7.43 | +322 | Preg. 48 hrs. |
| B15R1 | lft.<br>rt. | 7.37 - 7.45<br>7.37 - 7.35 | 0.08<br>0.02 | 7.41<br>7.36 | | Preg. 55 hrs. |
| B15R2 | lft. | 7.62 - 7.58 | 0.04 | 7.60 | | Preg. 55 hrs. |
| For pH mean, 6 = 0.082 Average = | | | 0.04 | 7.46 | +289 | |

Eh observations were made as in the preceding experiments. The average H-ion concentration was similar to that obtained during late metoestrum in the preceding series.

## DISCUSSION

*Possible factors influencing pH and Eh fluctuations of the uterine fluids.*—The maximum alkalinity of the uterine fluids was observed during late oestrus and early metoestrum, a period characterized by extensive vacuolar degeneration of the mucosa. The period of relative acidity of the uterine fluids during pro-oestrum and early oestrus is accompanied by rapid growth of the mucosa and active secretion by the uterine glands and epithelium. The secretions and exudations accompanying changes of the uterine mucosa and glands may account for the observed fluctuations in the acidity of the uterine fluids.

That a general condition of acidosis in an animal does not influence the H-ion concentration of uterine secretions has been demonstrated by Aasland (1932). However, the fact that the pH of the right and left uterine horns of Mouse I differed widely during the last 5 days of observation, while the pH curves for both horns fluctuated synchronously, raises the question whether the general metabolic state of the uterus may not be a factor influencing the H-ion concentration of the uterine fluids. It is significant, in this respect, that 24–40 hours prior to oestrus, the metabolic rate, as measured by the oxygen consumption of the uterus of the rat and mouse, is increased 300–400 per cent (Khayyal and Scott, 1931). It is possible that the increased activity of the mucosa cells during pro-oestrum, pseudopregnancy, and deciduoma formation may result in an increase in the $CO_2$ tension in the uterine lumen, which would tend to lower the pH of the uterine fluids at times coincident with the observed periods of increased acidity.

The development and growth of deciduomata is rapid, as is their degeneration. The acid condition and the low oxidation-reduction potential associated with the occurrence of deciduomata may arise from the pronounced metabolic activity of the decidual cells which are relatively isolated from the buffering and poising action of the circulating fluids.

The lack of complete correlation between the maxima and minima of the pH curves with the oestrus stages, as indicated by the vaginal smears, is believed to be the result of three factors: (1) the occurrence of some stages of the oestrus cycle between observations; (2) the animals were studied at varying times relative to the onset of the stage of oestrus indicated by the vaginal smear; (3) the vaginal smear is not an infallible index of the ovarian-uterine condition.

The pro-oestrus stage and the late metoestrus stage were most frequently missed, with the result that the low pH value preceding oestrus was, in such cases, associated with either a dioestrus smear or an early oestrus smear; and the pH minimum occurring after oestrus was associated with a stage IV smear. Different pH values of uterine fluids were often found in early and late periods of the same stage of oestrus. The importance of the time element and the lack of correlation of physiological oestrus with a definite "vaginal" oestrus is in-

dicated in that "heat," the "physiological" oestrus, may occur in pro-oestrum 3 hours before the appearance of cornified cells in the vagina or as late as 20 hours after their appearance (Long and Evans, 1922).

That the vaginal smear is not always an exact index of the ovarian uterine condition is supported by work of Hemingsen (1932), Clauberg (1931), and others. Hemingsen lists twenty-three cases where oestrus phenomena were exhibited in rats whose vaginal smear was dioestrus, late dioestrus, or late metoestrus. Clauberg, from a comparison of vaginal smears with histological studies of the uterus and ovary, concluded that the vaginal smear does not always afford an unquestionable index of the condition of the ovary, uterus, or even the vaginal epithelium itself.

In view of the possible lack of complete synchronization between the "vaginal oestrus," as indicated by the vaginal smear method, and the "uterine-ovarian oestrus," perhaps a clearer picture of the pH changes accompanying oestrus in the uterus may be had by considering the rise and fall of the pH curves without exact reference to the individual vaginal smears. If this is done, the average of the maximal pH values, usually occurring in early metoestrum in the rat series, is pH $7.67 \pm 0.11$ and in the mouse series pH $7.46 \pm 0.14$. The pH minima preceding oestrus average to pH $7.24 \pm 0.10$ in the rat and pH $7.11 \pm 0.12$ in the mouse, while the pH minima succeeding oestrus average to pH $7.21 \pm 0.11$ in the rat and pH $7.21 \pm 0.13$ in the mouse.

*Pseudopregnancy induction.*—Stimulation of the cervical canals of rats and mice during stages I, II, or III (e) of the oestrus cycle can be expected to interrupt the normal cycle and induce the condition of pseudopregnancy (Long and Evans, 1922; and Jongh, 1933). The smear records of the animals used in the present experiment show that this expectation was fulfilled in many cases.

It appears that only one mouse became pseudopregnant as the result of the rather vigorous stimulation of the cervical canals during the course of these observations. However, since a deciduoma was found in the left uterine horn of a second mouse, it must be concluded either that in this second animal the pseudopregnant condition developed or that a deciduoma was induced both to develop and

persist during normal oestrus cycles. It is the more likely that the condition of pseudopregnancy did not sufficiently suppress the cyclic rhythm of the ovaries (or ovary) to eliminate the periodic cornification of the vagina.

In the rat series, six definite cases of pseudopregnancy and a doubtful seventh developed, i.e., 75 per cent of the animals became pseudopregnant. This percentage is slightly above that obtained by Long and Evans (1922) and Meyer, Leonard, and Hisaw (1929) in unanesthetized animals. The latter authors report that lightly anesthetizing the animals with ether, nitrous oxide, or ethylene anesthesia decreased the percentage of pseudopregnancies resulting from artificial stimulation of the cervix to 10.4, 21.7, and 33.3 per cent. It is probable that in the difference of degree and duration of stimulation of the cervix lies the explanation of the discrepancy that exists between the results reported by Meyer *et al.* and those listed above.

*Significance of pH and Eh findings in relation to spermatozoa and eggs.*—Experiments *in vitro* on horse spermatozoa (Yamane and Kato, 1928) and on rabbit spermatozoa (Carter, 1932) have shown that mammalian sperm are very sensitive to small changes from the optimum H-ion concentration of the medium, which is between pH 7.2–7.4 for horse sperm and pH 7.9 for rabbit sperm. The alkaline nature of the uterine fluids during oestrus in the rat and mouse suggests that the uterine fluids may incite and favor the activity of spermatozoa *in utero*. The oxidizing nature of the uterine fluids (indicated by the Eh values obtained in the uterine lumen) probably also favors spermatozoan activity in the uterus.

In the modern concept of biological oxidations the oxidation-reduction potential of a medium plays an important rôle in cellular oxidation. The importance of the oxidation-reduction potential to developing sea-urchin eggs has been shown by Reiss and Vellinger (1929), who found that lowering the Eh of sea water to about +188 mv. (sea water = approximately +248 mv.) resulted in about 100 per cent cessation of development. The oxidation-reduction potentials of uterine fluids in the rat and mouse at the time of entrance of the egg into the uterus are well above those essential for the developing sea-urchin egg and indicate a system strongly oxidizing in action.

The pH curves of the pseudopregnant animals indicate an increase in the H-ion concentration of the uterine fluids at a time when the egg, in a normal pregnancy, would be free in the uterine lumen. This is especially true in the six pseudopregnant rats whose pH curves 4–6 days after oestrus show an average of pH $7.14 \pm 0.17$.

From Defrise's (1933) experiments on the culture of the rat egg *in vitro*, it appears that the increased acidity of the uterus in the early dioestrum and pseudopregnancy presents a more favorable environment for the developing egg than that of the oestrus uterus.

The period of implantation is coincident with the time when the endometrium is "sensitized" to deciduoma formation upon injury to the mucosa. It may be that the increased H-ion concentration observed in the uterine fluids during this period is either a necessary condition for the development of the decidua or merely a concomitant or resultant condition arising from the pronounced histo-physiological changes taking place at this time in the mucosa.

While the pH values obtained for the normal uterine fluids in the rat and mouse were higher than those of the acidified Ringer's solution used for oölemmal removal *in vitro* (Nicholas and Hall, 1934; Hall, 1935), the low pH obtained in the deciduomata indicates that the egg during normal development is subjected to an acid environment during implantation. As the decidua develops around the implanting egg and as the metabolic activities of the dividing blastocyst increase, the fluid bathing the blastocyst may become sufficiently acid to be a factor in the removal of the oölemma.

## V. SUMMARY AND CONCLUSIONS

1. The uterine fluids, studied *in situ* in the living, uninjured rat and mouse, vary in H-ion concentration and oxidation-reduction potential during the normal oestrus cycle, pseudopregnancy, and early pregnancy.

2. The uterine fluids in the rat are in general more alkaline and show greater fluctuations during the oestrus cycle than those in the mouse.

3. In both the rat and mouse the occurrence of oestrus is associated with a decrease in the H-ion concentration of the uterine fluids, which reaches a maximum generally in early metoestrum. About 3

days after oestrus in the rat and slightly before this in the mouse, the H-ion concentration of the uterine fluids increases to a value approximately equal to that at pro-oestrum.

4. The oxidation-reduction potentials in the rat uterus were generally higher than those obtained in the mouse uterus. The evidence for cyclic variation in the oxidation-reduction potential during the oestrus cycle is not conclusive, although the values obtained during metoestrum were consistently low.

5. A decrease in the alkalinity of the uterine fluids was found during the fourth to sixth day of pseudopregnancy, which suggests that the decreased alkalinity is associated with the "decidual reaction" and that it may be of importance to the normal development and implantation of the egg.

6. The development and degeneration of deciduomata was characterized by a definite acidity of the uterine contents.

7. Two "regular" oestrus cycles, as indicated by vaginal smears, occurred in a mouse whose uterus contained a large deciduoma when examined on the eleventh day of "pseudopregnancy."

8. The acidity and the low oxidation-reduction potential which develop in the lumen of excised uteri demonstrate that such observations give an inaccurate and misleading conception of the conditions prevailing within the uterus of the living animal.

9. Cyclic variations in the glandular and metabolic activity of the uterine mucosa, correlated with the structural changes of the uterus, are suggested as possible factors influencing the variations in the H-ion concentration of the uterine fluid.

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# ACTIVITY OF THE ADDUCTOR MUSCLE IN OYSTERS[1]

(Three figures)

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INVESTIGATIONS on the activity of the adductor muscle of lamellibranchs have been primarily from the point of view of feeding habits. In the oyster there is only one adductor muscle, morphologically the posterior, and this consists of a posterior rather crescent-shaped portion of striated muscle partially surrounding the more anterior plain muscle. The function of the striated portion is to cause the two valves to approach closer together. Its effect on the position of the shells is dynamic in contrast to that of the plain muscle, which serves to maintain any given position of the valves against the pull of the hinge ligament.

It was concluded by Kellogg (1915) that lamellibranchs feed only when the water pumped by the gills is relatively clear and that, when the water contains a large quantity of suspended matter, particles of silt and other material are altogether discarded and do not enter the digestive tract. Nelson (1921, 1922, 1923) made a study of the actual ingestion of food by the oyster under different conditions of turbidity of the water and reached the conclusion that the amount of silt in the water had little to do with the actual feeding process. He found that the adductor muscle contracted more frequently in water containing a large quantity of suspended material than in clear water, and used his findings in a study of the rate of feeding under different conditions. His method was to keep records of the shell movements and to estimate the rate of feeding from the turbidity of the water and the frequency of shell closures which result in the discarding of accumulated material.

Galtsoff (1928) called this method into question after a series of experiments in which he found that partial closures frequently occur without any obvious discharge of material. He also pointed out that contractions of the adductor muscle of a periodic nature were at

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times to be observed. However, the conclusions reached by Nelson concerning the rate of feeding of oysters at different temperatures are not greatly at variance with those obtained by Galtsoff using a more accurate method.

Recently Orton (1935) put forward the hypothesis that the function of the otherwise unexplained contractions of the striated portion of the adductor muscle is to relieve fatigue in the plain catch-muscle portion. This idea does not appear to be in harmony with what is known of the physiology of plain muscle, though it is not inconceivable that in the oyster plain muscle may require an occasional resting period. It was with the purpose of attempting to reach some conclusion as to the nature and function of the frequent partial closures of the oyster that the following observations were made.

## MATERIAL AND METHODS

Already at hand were a great many kymograph records of shell movements of the Japanese oyster, *Ostrea gigas*, which had been made for different purposes. Not only do these records show the degree of openness of the shell and the movements due to the adductor muscle, but they show also the relative rate at which water is pumped. The method of recording the rate of flow of water has already been described (Hopkins, 1933, 1935). It consisted of a small cone attached to a system of levers and placed so that water from the cloaca struck the cone, causing a movement of the levers which recorded the relative rate of flow on the same kymograph papers on which shell movements were recorded. Some of the records were made at approximately constant temperature; others, at temperatures varying from about 2° C. to 30° C.

More direct study of the adductor muscle was made on small spat, 0.5–2.0 cm. in diameter, of *Ostrea lurida*, which had been caught on plane glass plates. The plates were put directly upon the stage of the microscope and the shells of the spat scraped clean so as to permit observation by transmitted light.

## OBSERVATIONS

A study of the kymograph records indicates that partial closures due to contractions of the adductor muscle may be classified into

three groups according to the resulting discharge of water from the branchial and cloacal chambers. Characteristically, when an oyster is about to make a partial closure, the borders of the mantle arrange themselves so as to leave an opening between them in one particular locality while remaining otherwise close together, as described by Galtsoff (1930) for spawning by the female. In this manner, a strong current is ejected from a certain place, normally resulting in the discharge of waste or rejected material.

Some contractions involve a discharge primarily from the branchial chamber or some small portion of it. Other contractions are concerned almost entirely with the cloacal chamber, resulting frequently in the discharge of feces. A third type may be called non-

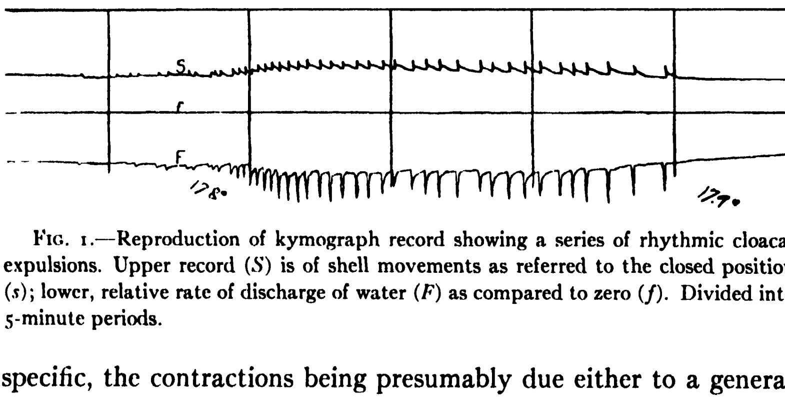


Fig. 1.—Reproduction of kymograph record showing a series of rhythmic cloacal expulsions. Upper record (*S*) is of shell movements as referred to the closed position (*s*); lower, relative rate of discharge of water (*F*) as compared to zero (*f*). Divided into 5-minute periods.

specific, the contractions being presumably due either to a general stimulus, such as vibrations or change in the intensity of the light, or to internal causes; and the borders of the mantle are not arranged to concentrate the discharge at any particular place.

A study of the kymograph records mentioned above clearly shows the marked distinction between the results of the different contractions. In cases in which the discharge was from the cloaca, the levers recording the rate of flow were violently disturbed; while if the discharge was from the branchial chamber, little or no effect was to be observed save that the rate of flow of water from the cloaca was suddenly reduced (Fig. 1). In the case of the non-specific contractions, a relatively slight disturbance of the lever recording rate of flow is to be observed.

*Cloacal expulsions.*—While contractions of the muscle occur in general with little apparent system, at times there occur periods of definitely rhythmic contractions which result in expulsions of water from the cloacal chamber. In Figure 1 is a portion of a kymograph record showing such a series. Typically, the contractions begin at a relatively slow rate, which very rapidly increases to a maximum and then slowly diminishes until the contractions cease. At the beginning of such a series, the contractions are small in amplitude, increasing gradually. A series of periodic expulsions may occupy only a few minutes or may be prolonged for an hour or more, as shown by Figures 2 and 3. One of the series shown graphically in Figure 3 is very regular; the other suggests a possible overlapping and fusion of two series. The figures represent extremes of variation in periodicity of this type of contraction.

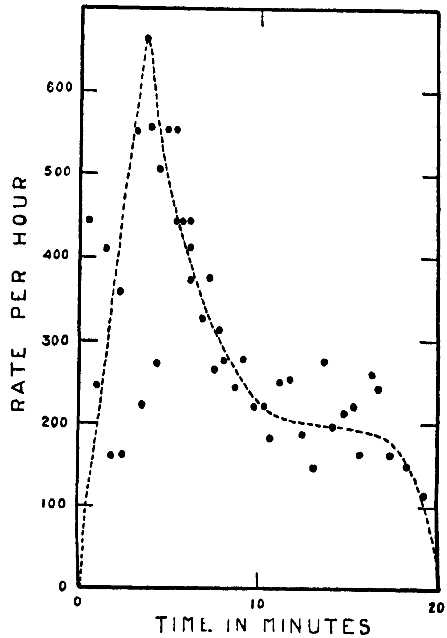


FIG. 2.—Frequency of rhythmic contractions of adductor muscle of *Ostrea gigas* determined from time between consecutive contractions. Same series as in Figure 1.

Apparently, the only important difference between the various series is in the duration of the activity at the most rapid rate. It is probable that the function of the series of rhythmic cloacal expulsions is the discharging of fecal material from the cloacal chamber. In a great many cases this has been actually observed, although such series may at times occur when there is no observable solid discharge. It is also true that defecation may take place without accompanying periodic cloacal expulsions. However, in cases of periodic contractions the borders of the mantle are so arranged as

to concentrate the discharge through the cloacal chamber. The form of the curves of Figures 2 and 3 describing these series of expulsions suggests what may be considered an internal origin rather than an external one, such as might be due to irritation by some foreign body. One may think of a series of nervous discharges arising in a portion of the digestive system through which waste material is passing.

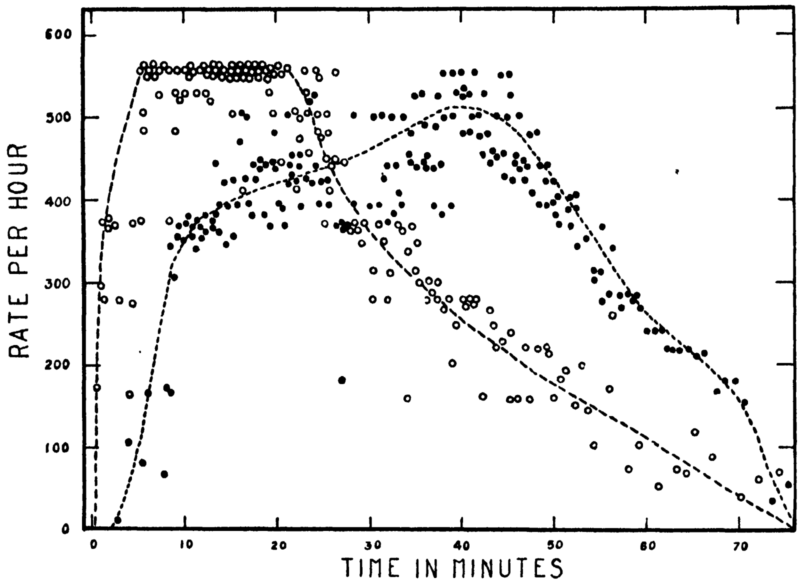


FIG. 3.—Frequency of rhythmic contractions of adductor muscle of *Ostrea gigas* determined from time between consecutive contractions. Two separate series.

Kymograph records of series of rhythmic partial closures, such as that shown in Figure 1, resemble those reproduced by Nelson (1922) and by Galtsoff (1930) of shell movements during discharge of eggs by *Ostrea virginica*. In the case of the spawning reaction, however, the discharge of eggs is from the branchial (mantle) chamber. Spawning by the male appears to involve no activity of the adductor muscle, for the sperms are released directly into the excurrent stream of water. That the periodic partial closures described above were not due to discharge of eggs is shown by the fact that during several experiments specimens which frequently exhibited such closures spawned as males, the two activities never coinciding.

Galtsoff (1930) also noted that adrenalin causes rhythmical contractions of the adductor muscle.

*Other expulsions.*—Although a great many kymograph records have been carefully measured and studied, there appears to be no obvious system in the occurrence of adductor contractions other than those described above. In some series of experiments the specimens remained for several days in the same water artificially circulated. In such cases there were few or no particles entering the water which might irritate the specimen, but yet occasionally there would be a sudden partial closure. In some cases these were non-specific, and in some they resulted in a concentration of discharged water from either the branchial or the cloacal chamber. The experiments were made in the laboratory, and it was often noticed that a sudden vibration would cause such a reaction. It is probable that most, if not all, of these expulsions are traceable to some external stimulation—either light, vibration, suspended matter in the water, or an accumulation of secreted mucus. In favor of such a view is the fact that the same specimen on consecutive days in the same water and under apparently almost identical conditions varies tremendously in the frequency of the contractions (Table I). Also, the records indicate that contractions are more frequent during a short time following a change of water which would contain more suspended matter. This is in accord with Nelson's conclusions. Furthermore, there appears to be no correlation between the degree of openness of the shell and the frequency of these contractions. Also, attempts to correlate the frequency of the contractions with the salinity of the water resulted in failure, save that a salinity below about 12 per thousand results in inhibition of the contractions as well as of the flow of water.

In experiments carried on with the temperature ranging from about 2° C. to over 30° C., the frequency of these non-specific contractions appears to have no direct relationship with temperature. On the other hand, periods of rhythmic cloacal expulsions definitely occur more frequently at temperatures above 15° C. than below, as would be expected if due to activity of the digestive system.

*The adductor muscle of spat.*—The spat shells of *Ostrea lurida* do not become greatly calcified until the diameter becomes over a centimeter. Spat caught upon glass plates were therefore excellent

material for direct observation. Studied by transmitted light with strong illumination the two sections of the adductor muscle are readily observable. When the spat is at rest, open and pumping water, the striated portion is very transparent while the plain muscle is relatively opaque. When the striated portion contracts, it becomes suddenly opaque and definitely darker than the plain muscle. As relaxation occurs, one readily sees that the all-or-nothing principle applies to this muscle, for the fibers return sepa-

TABLE I

FREQUENCY OF NON-PERIODIC CONTRACTIONS OF ADDUCTOR MUSCLE OF *Ostrea gigas* (SPECIMEN II) AT 17°–19° C.

| Date 1932 | Salinity (Parts per Thousand) | Total Time (Min.) | No. of Contractions | Av. No. of Contractions per Hour |
|---|---|---|---|---|
| 11/28 | 28.87 | 185 | 41 | 13.3 |
| 11/29 | 22.70 | 385 | 128 | 20.0 |
| 11/30 | 22.70 | 335 | 40 | 7.2 |
| 12/1 | 28.74 | 355 | 90 | 15.2 |
| 12/2 | 28.74 | 390 | 24 | 3.7 |
| 12/3 | 28.74 | 160 | 9 | 3.4 |
| 12/4 | 25.08 | 325 | 157 | 29.0 |
| 12/5 | 25 08 | 250 | 19 | 4.6 |
| 12/7 | 28.94 | 310 | 16 | 3.1 |
| 12/8 | 17.85 | 145 | 18 | 7.4 |
| 12/9 | 17.85 | 180 | 22 | 7.3 |
| 12/12 | 17.83 | 265 | 15 | 3.4 |
| 12/13 | 17.83 | 235 | 19 | 4.9 |
| 12/14 | 17.83 | 120 | 10 | 5.0 |
| 12/15 | 17.83 | 80 | 11 | 8.3 |
| 12/16 | 17.83 | 135 | 6 | 2.7 |
| 12/17 | 17.83 | 130 | 23 | 10.6 |
| 12/19 | 27.27 | 265 | 35 | 7.9 |

rately to transparency. As more of the fibers relax, the striated muscle takes on a splotched appearance, with some portions clear and others opaque.

Contraction appears to be brought about by synchronous action of all the fibers which take part in the process. The darkening is sudden, with no indication of progression. Relaxation, however, generally begins with the fibers adjacent to the inner border of the crescent-shaped muscle and passes across as an irregular wave. In the kymograph record reproduced (Fig. 1) it will be noted that the partial closures of the valves are quick, the record line being vertical.

Relaxation begins quickly but requires from a few seconds up to about a minute to become complete. Observations on the spat muscle demonstrate that this is due to the time required for all fibers of the striated muscle to relax.

Spat have been observed under the microscope in this manner for long periods of time, and the occasional contractions observed. If Orton's (1935) idea is correct that rhythmic contractions of the striated portion have the function of relieving fatigue in the plain muscle, one would expect to observe contraction of the striated portion as frequently when the oyster is closed as when open to any degree. However, in spite of many attempts, no such contraction has been observed when a specimen was closed. One would think that fatigue of the plain muscle would be at least as great when working against the entire strength of the hinge as when supporting only a small portion of this burden. Although one cannot state that contraction of the striated muscle does not actually result in relieving fatigue in the plain muscle, it would not appear to be entirely correct to say that such is a function of this activity.

## DISCUSSION

Partial closures of the valves of the oyster are sometimes definitely periodic, sometimes apparently only occasional and unorganized. For hours at a time a specimen may remain open, pumping water vigorously, while the valves remain motionless. At other times partial closures occur more or less frequently. That at least some of the occasional closures are the result of external stimulation by vibration, change in the intensity of light, suspended matter in the water, and such factors, is well established, although it is not improbable that some of them may owe their origin to internal causes. The obvious function of these sudden partial closures is to discharge particles of silt, mucus, or other material from the surfaces of the mantle or gills; and one may observe the borders of the mantle become so arranged in advance of the closure as to direct most of the discharged water from some particular place. Even in cases in which there is no observable solid discharge, the directive action of the borders of the mantle may be seen.

On the other hand, there occur series of rhythmic closures the frequency of which varies according to a relatively definite system. In

all cases studied, these result in a discharge of water through the cloaca, often with accompanying observable defecation. It appears most probable from the nature of the rhythmicity that the origin of this activity is within the digestive system through which material is passing.

Partial closure of the valves is due to contraction of the striated component of the adductor muscle, while the smooth catch-muscle component maintains the valves in any given position. Physiological studies, which need not be reviewed here, have shown that catch-muscle expends little or no more energy when contracted than when relaxed. Orton (1935) questioned such conclusions by the hypothesis that rhythmic contractions of the striated component of the muscle of the oyster have the function of relieving fatigue in the catch-muscle. On theoretical grounds alone his idea would hardly appear to be tenable.

It was possible, however, to obtain some direct evidence on the matter by means of a microscopic study of small, relatively transparent oysters by transmitted light. Contractions of the striated portion of the muscle results in a sudden change from transparent to opaque; and while contractions were often observed when the valves were open, none could be seen when the valves were closed. Under the latter condition fatigue of the smooth component would presumably be at least as great as when the valves are open. Orton's hypothesis, which is an attempt to explain the origin of contractions of the striated muscle without direct observation on the muscle itself, does not appear to stand upon a very solid foundation in view of the observations described above as well as theoretical considerations.

The relaxation of the striated component of the adductor muscle furnishes an excellent example for direct observation of the application of the all-or-nothing principle in the individual relaxation of fibers.

## SUMMARY

1. Contractions of the adductor muscle producing partial closure of the shell fall into three classes according to whether water is discharged (*a*) from the mantle chamber, (*b*) from the cloacal chamber, or (*c*) from both without direction.

2. Frequently there occur rhythmic partial closures resulting in expulsion from the cloaca. Such series are analyzed graphically; and it is considered probable that their function is the elimination of feces, although their origin appears to be internal.

3. Occasional contractions occur which may concentrate discharged water from either branchial or cloacal chamber. These are not obviously correlated either with the degree of openness of the valves, with temperature, or with salinity of the water, but are most frequent when the amount of suspended matter in the water is greatest. They appear to be due primarily to external stimulation, though some may have an internal origin.

4. By observing living spat by transmitted light the activity of the striated portion of the adductor muscle was studied. When contracted, it is opaque; when relaxed, transparent. Contraction is quick; relaxation, slow, involving a few fibers at a time. The application of the all-or-nothing principle was readily obvious.

5. In spat the striated muscle could never be observed to contract while the valves were closed, suggesting that Orton's (1935) theory that periodic contractions of this muscle serve to relieve fatigue in the plain muscle may not be well founded.

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# A COMMON FACTOR IN PLANARIAN AND MAMMALIAN NUTRITION[1]

(Three figures)

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THE authors have carried on a prolonged series of investigations using the planarian worm to test the nutritional value of guinea-pig tissues (Bahrs and Wulzen, 1936). The guinea pigs were fed diets constructed for the study of the different vitamins, and their tissues became the sole diet of groups of planarian worms. It was found that liver, heart, and kidney derived from these guinea pigs produced, with only one exception, a dietary disease in planarians even though the recognized vitamins were adequately supplied in the diet given the animals furnishing the tissues. The one exception occurred when a supplement of green feed (the authors used kale and grass) had been included in the diet of the guinea pigs. The tissues from the animals receiving the green-feed supplement produced normal growth in the planarians, and the worms were entirely healthy. In certain experiments ample supplies of vitamin C were given the guinea pigs in the form of orange juice and tomato juice; but this in no way diminished the disease-producing power of the liver, kidney, and heart tissues furnished by those animals which did not receive kale or grass.

This protective factor for planarian worms seemed so definitely distinct from other recognized nutritional factors that it was given a name, factor *pl.* For convenience of reference this terminology is followed throughout the present paper.

Although the guinea pigs furnishing tissues which produced disease in planarians appeared normal during the course of the ex-

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periments, a period of about $2\frac{1}{2}$ months, we thought it advisable to endeavor, by means of a more prolonged feeding period, to emphasize possible latent disturbances in the guinea pigs to a point where they might become overt.

Three prolonged series of experiments were completed on slightly different basal diets, Diets, 10, 11, and 12.

DIET 10

| | Parts |
|---|---|
| Baked milk powder | 25 |
| Ground barley | 42 |
| Bran | 23 |
| Yeast* | 5 |
| Cod-liver oil | 3 |
| NaCl | 1 |
| $CaCO_3$ | 0.5 |
| Ferric ammonia citrate | 0.5 |
| KI | 0.002 |

(Given individually, 1 cc. of cod-liver oil, 2 drops viosterol, twice per week)

DIET 11

| | Parts |
|---|---|
| Baked milk powder | 20 |
| Ground oats | 15 |
| Ground barley | 28 |
| Bran | 23 |
| Yeast | 5 |
| Baked alfalfa meal | 4 |
| Cod-liver oil | 2 |
| NaCl, iodized | 1 |
| $CaCO_3$ | 1.5 |
| Ferric ammonia citrate | 0.5 |

(Viosterol, 1 drop per cubic centimeter of cod-liver oil)

* We wish to express our sincere thanks to Mead Johnson and Company for generous supplies of yeast, cod-liver oil, and viosterol.

DIET 12

| | Parts |
|---|---|
| Ground barley | 30 |
| Ground oats | 27.75 |
| Bran | 16 |
| Alfalfa, dried | 20 |
| Yeast | 5 |
| NaCl, iodized | 1 |
| Cod-liver oil | 0.25 |

(Viosterol, 1 drop per cubic centimeter of cod-liver oil)

### FIRST SERIES OF EXPERIMENTS

Diet 10 was administered to six groups of guinea pigs. Supplements to this diet were given as follows: Group I, 14 females, 15 cc. fresh tomato juice per animal per day; Group II, 14 females, 20 g. fresh kale per animal per day; Group III, 7 females, 20 g. fresh kale and 15 cc. fresh tomato juice per animal per day; Group IV, 13 males, 15 cc. fresh orange juice per animal per day; Group V, 13 males, 20 g. fresh kale per animal per day; Group VI, 8 males, 20 g.

fresh kale and 15 cc. fresh orange juice per animal per day. All animals were bedded in straw and ate of it abundantly. Table I shows the average percentage gains of the groups. It is evident that the animals in the groups which received kale showed much higher percentage weight gains than did the animals in the groups which received no kale.

During the course of the experiment the animals in the groups receiving no green feed (Groups I and IV) gradually sickened and died, and the feeding continued until all the animals had succumbed —a period of 9 months. At the end of the experiment, all animals

TABLE I*

AVERAGE PERCENTAGE GAINS IN WEIGHT OF ANIMALS RECEIVING BASAL DIET 10, WITH OR WITHOUT ADDITION OF FRESH KALE

| Group | No. of Animals | Sex | Diet Supplement | Av. Time (Weeks) | Percentage Av. Wt. Gain |
|---|---|---|---|---|---|
| I | 14 | ♀ | Tomato | 20 | 65 |
| II | 14 | ♀ | Kale | 20 | 114 |
| III | 7 | ♀ | Tomato and kale | 20 | 115 |
| IV | 13 | ♂ | Orange | 16 | 38 |
| V | 13 | ♂ | Kale | 16 | 59 |
| VI | 8 | ♂ | Orange and kale | 16 | 57 |

* The lower gains of the groups of male animals as compared with the females were probably due to the fact that the males were very active and fought continually, while no fighting occurred among the groups of females.

in all groups which had received green feed (Groups II, III, V, and VI) were alive and in splendid condition. Since Group III received tomato juice and Group VI orange juice, it is probable that these juices had no intrinsic harmful effect on the animals.

In all cases where sickness occurred, a distinctive syndrome characterized the development and course of the disease. An early sign was the lowering of muscle tone so that the animals remained flaccid when handled. They developed difficulty in moving about the pen, because the hind limbs could not be manipulated well and became tangled in the straw. The righting time was considerably prolonged in these animals, and at length they could no longer right themselves when placed on their backs. The development of the scratch reflex was pronounced. In the normal controls any amount of tickling

failed to elicit the scratch reflex of the hind legs; but the subnormal animals first showed great restlessness under tickling along the back and sides and, as the disease progressed, they gave the scratch reflex at once on slight contact. In some individuals the reflex became so violent as to amount to convulsive seizures. There was also an involvement of the eyes, which was analyzed more carefully in connection with the second series of experiments and will be discussed later in the paper.

At a time when the disease was well developed, hemoglobin determinations were made on animals from five of the experimental groups. The Haskins-Sahli method was used in which 100 per cent is equivalent to 13.8 g. hemoglobin per 100 cc. blood. The results (in

TABLE II

BODY TEMPERATURE OF ANIMALS SHOWING SYMPTOMS OF FACTOR *pl* DEFICIENCY, COMPARED WITH THAT OF ANIMALS SUPPLIED FACTOR *pl* IN THE FORM OF FRESH KALE

| Group | No. of Animals | Diet Supplement | Av. Body Temp. at 9:00 A.M. | Av. Body Temp. at 8:00 P.M. |
|---|---|---|---|---|
| II and V | 15 | Kale | 100.7° F. | 102.2° F. |
| IV | 10 | Orange | 99.2 | 100.6 |
| I | 6 | Tomato | 99.8 | 100.7 |

percentage) were as follows: Group I, basal+tomato, 113, 124, average 118; Group II, basal+kale, 99, 100, average 99.5; Group III, basal+tomato+kale, 95, 99, average 97; Group IV, basal+orange, 115, 99, 100, 108, average 105.5; Group VI, basal+orange+kale, 98, 110, 94, 103, average 101.2. From these results it appears that the animals which received no kale and were showing marked signs of the deficiency disease manifested no diminution in hemoglobin.

We found that the body temperature of guinea pigs which showed definite symptoms of factor *pl* deficiency was lower than that of the controls. Table II shows the daily body temperature rhythm of animals from several of the experimental groups.

Guinea pigs manifesting symptoms of factor *pl* deficiency showed a marked fall in body temperature when subjected to a colder environment. Animals fed the same basal diet supplemented with fresh

kale suffered a very slight loss in body temperature when exposed to the same degree of cold. Table III illustrates this point.

Eventually the diseased animals could not move about the pen at all but lay on their sides with their legs extended. The joints were not swollen, nor was the hair rumpled; but the hair seemed thinner and coarser than in the healthy animals. After the animals were prostrate, death from inanition naturally supervened.

TABLE III

COMPARISON OF LOSSES IN BODY TEMPERATURE, WHEN EXPOSED TO COLD, OF ANIMALS SHOWING SYMPTOMS OF FACTOR *pl* DEFICIENCY AND ANIMALS SUPPLIED WITH FACTOR *pl* IN THE FORM OF FRESH KALE

| No. of Animals | Diet Supplement | Time of Exposure to Cold (Min.) | Temp. | Average Loss in Body Temp. of Animals | |
|---|---|---|---|---|---|
| | | | | Without *pl* | With *pl* |
| 1 | Orange | 20 | 34° F. | 3.1° F. | .......... |
| 2 | Kale | 20 | 34 | .......... | 0.1° F. |
| 2 | Orange | 30 | 34 | 3.1 | .......... |
| 1 | Kale | 30 | 34 | .......... | 2.0 |
| 2 | Orange | 30 | 40 | 2.1 | .......... |
| 1 | Tomato | 30 | 40 | 5 1 | .......... |
| 1 | Tomato | 20 | 40 | 4.2 | .......... |
| 3 | Kale | 30 | 40 | .......... | 0.46 |
| 3 | Orange | 20 | 45 | 2.6 | .......... |
| 1 | Kale | 20 | 45 | .......... | 0.3 |
| 2 | Orange | 30 | 45 | 2.7 | .......... |
| 5 | Kale | 30 | 45 | .......... | 0.6 |
| 2 | Orange | 20 | 48 | 3.3 | .......... |
| 1 | Kale | 20 | 48 | .......... | 0.1 |

We undertook to feed some of these individuals with a pipette to prevent starvation. The basal diet regularly used was made into a paste with water and was administered three times a day. The animals seemed very hungry and ate their food well. The orange and tomato juices were given as usual.

Those animals which were kept alive by pipette feeding and which did not receive kale gradually stiffened until both fore and hind limbs were thrust caudally in extreme extension. The toes showed a tendency to flex. There was some power of movement left in hip and shoulder joints. The legs were very strongly adducted, and the

posterior limbs could not have been spread apart without tearing the adductor muscles. The head was drawn back so that the occiput was pressed against the shoulder blades. It was impossible to press the head downward, and the whole body was so stiff as to be completely immobilized (see Figs. 1, 2, and 3). Even these very stiff animals ate well from pipettes, chewing and swallowing normally.

FIG. 1.—Live guinea pigs, fed Basal Diet 10 and orange juice for 6 months, until they became unable to move. They were then fed the same diet by hand for about 2 months. Their bodies were completely immobilized, the head and legs having the permanent forced positions shown.

The feces appeared normal also until the animals were about to die, when they became soft. For the last day or so before death the animals failed to eat well.

The following reflex was present: If light pressure toward the body was applied to the hind feet, the fore feet were thrown into a somewhat caudal position, and the muzzle moved slightly dorsad.

The eyes were open constantly, and no stimulation would make

them close. In the most developed cases they appeared opalescent. Hearing was very acute, and the animals twinged at any sharp sound when normal controls gave no sign. In one case when an injection was given in the posterior region of the abdomen, a cry of

FIG. 2.—A normal guinea pig lying on its back. Note spread of legs and position of head

pain was elicited. Terminally a coma developed, so that it was hard to rouse the animals to eat.

At autopsy, the liver often seemed hypertrophied and had a liquefied appearance, the hepatic lobules being indistinct. Others showed the hepatic lobules more distinct than in the normal animal. The liver was commonly mottled and often pale, giving the appearance of fatty degeneration. There were no conspicuous hemorrhages. The spleen appeared contracted and brownish or hypertrophied. The

ventricular tissue was soft and flabby. The skeletal muscles did not relax at all after death, the body maintaining the rigid position of life. There was extreme muscular atrophy. The muscles appeared grayish or yellowish white in color, of a waxy luster, and hard. In

Fig. 3.—One of the guinea pigs of Figure 1, lying on its back. Note the strong adduction and extension of the legs and the dorsally thrust head, in contrast to the normal positions shown in Figure 2.

portions they glistened because of the predominance of fibrous tissue. One case examined showed that in the adductor muscle mass a heavy tendinous band extended from the pubic bone to the inferior end of the linea aspera, with no intervening muscle. It was considerably shorter than the normal muscle would have been, so that the femur was strongly adducted and completely immobilized.

Skeletons prepared from diseased and control animals showed definite differences. The bones of the diseased animals were much thinner and more translucent than the controls. This was particularly true of the scapula, innominate bone, ribs, and vertebrae. The edges of the scapula and iliac bone were irregular and very rough. The transverse processes of the vertebrae were very thin and flattened. The spinal column could be cut across easily with scissors. Where the long bones of the controls were smooth, those of the diseased animals often felt finely granular. The translucent appearance of the bones and their strong odor suggested the presence of fat. The bones of the controls had no such appearance or smell.

In two cases we found it possible to restore a thoroughly sick animal to health by the addition of macerated kale to the diet, or kale leaves fed by hand when the animal became stronger. After the apparent recovery of one animal the kale was removed from the diet and the animal again developed the characteristic symptoms of the disease. When it was close to death the kale was restored, and the animal brightened considerably and developed a good appetite. It was kept alive for 2 months apparently in good health, but the stiffened joints were never restored to the normal condition and the animal was entirely helpless. It finally died of acute indigestion.

Another animal was given kale after it had stiffened, and the supplementary kale feeding was continued for 5 months. During this time there was a very gradual improvement from complete immobility and helplessness. The anterior end of the animal recovered almost entirely, so that the anterior limbs were quite limber and the head could be moved normally. The posterior limbs were permanently stiffened and adducted, so that the animal had to be set upon its feet in order to stand. When so placed, it could pivot about to reach its food; and it ate abundantly. It was finally killed because it showed no further signs of improvement.

## SECOND SERIES OF EXPERIMENTS

Four groups of animals comprised the second series of experiments. They were given Basal Diet 11 with supplements as follows: Group VII, 13 females, 10 cc. fresh orange juice per animal per day; Group VIII, 10 males, 10 cc. fresh orange juice per animal per day;

Group IX, 7 females, 20 g. fresh kale per animal per day; Group X, 10 females, 6 cc. orange juice and 20 g. cooked kale per animal per day. This kale was soaked overnight in 2 per cent NaCl and was cooked in steam 1 hour. When the experiment had been in progress 3 months, the kale season was at an end and fresh alfalfa had to be substituted. The amount of alfalfa allowed each animal was 40 g.

The percentage gains in weight of the experimental groups at the fifteenth week of the experiment are shown in Table IV. It is evident

TABLE IV

AVERAGE PERCENTAGE GAINS IN WEIGHT OF THE ANIMALS GIVEN BASAL DIET 11 AND SUPPLEMENTS OVER A PERIOD OF 15 WEEKS

| Group | No. of Animals | Sex | Diet Supplement | Percentage Av. Weight Gain |
|---|---|---|---|---|
| VII.......... | 13 | ♀ | Orange juice | 46 |
| VIII......... | 10 | ♂ | Orange juice | 38 |
| IX........... | 10 | ♀ | Kale and alfalfa | 72 |
| X............ | 10 | ♀ | Cooked kale and alfalfa | 63 |

that at this time the animals which received no kale had fallen in weight considerably below those which received kale; they were manifestly weak, and their weakness progressed steadily. At length, just as in the previous series of experiments, they became unable to move their hind legs through the bedding straw.As soon as each animal reached this point, it was segregated and given individual treatment, with the object of either emphasizing or curing the diseased condition. When it was found that the animals subjected to these treatments were not eating as they should, the basal diet which they were receiving was made into a mash with water and was fed to them twice a day with a medicine dropper. Some abbreviated protocols follow:

## PROTOCOLS

*Basal diet and orange juice without further supplement:*

No. 301, female, Group VII. 4/30: Segregated because of stumbling in straw. 5/5: Wrists stiff; adductors are drawing legs together. 5/12: Legs stiff and thrust caudally. Helpless. Head drawn back onto shoulders. Still eats well from dropper but has no control over any part of body except mouth. 5/14: Dead.

Autopsy: Body rigid in all parts. Right heart much dilated. Liver enlarged, pale, and mottled. Lungs in good condition. Limb muscles hard and glistening in appearance, as if they were mostly tendon. Cutting away the muscles from the bones liberated the joints so that they could be flexed almost normally; thus the shortened muscles were the hindrance to joint mobility.

*Addition of fresh alfalfa:*

No. 277, male, Group VIII. 4/28: Segregated because of stumbling in straw. Fed by hand on basal diet mash. Given fresh alfalfa tips, which it could eat well. 5/3: Legs much better. Can now feed itself. 5/5: Apparently entirely cured. Dismissed.

No. 289, female, Group VII. 5/9: Segregated because of stumbling in straw. Given fresh alfalfa tips. 5/12: Can now run about pen almost normally, but is still weak and has not much appetite. 5/17: Very well in every way. Dismissed.

*Addition of calcium:*

No. 285, male, Group VIII. 5/7: Segregated because of stumbling in straw. Eyes show whitish exudate. Wrists stiff. Given injection of ½ cc. of 20 per cent calcium gluconate intramuscularly each day for 3 days; then 3 per cent of calcium lactate was added regularly to the basal mash. 5/14: Very weak; feeding by hand begun. Eating well. 5/17: Eyes very sore, filled with watery and milky exudate. Cornea opalescent. Legs very stiff. 5/22: Dead. Autopsy: Eyes have receded into socket and are suffused with exudate. Cornea dull. Eyeballs soft. Legs extremely stiff and strongly adducted. Liver pale and mottled and, when sectioned, appeared discolored around bile ducts. Auricles gorged with blood. Spleen shrunken and brownish. Formed feces in digestive tract. Had eaten well until a meal or two before death, when swallowing was difficult.

*Addition of viosterol:*

No. 223, male, Group VIII. 5/7: Segregated because of stumbling in straw. Given 15 drops of viosterol daily. 5/19: Can do nothing for itself. Legs stiffening. 5/22: Much worse but still able to eat. Eyes sinking back and quite watery. 5/23: Found dead. Autopsy: Legs moderately stiff at all joints. Much exudate, watery and milky, in eyes. Eyeballs soft. Liver pale and mottled; in section, discolored around bile ducts. Spleen shrunken and brownish. Kidney showed white infarcts. Formed feces in digestive tract. Had eaten from dropper up to very last. Lungs normal. Auricles gorged with blood.

*Addition of cod-liver oil:*

No. 314, female, Group VII. 5/9: Segregated because of stumbling in straw. Given 1 cc. cod-liver oil, containing 1 drop of viosterol, daily. 5/15: Became suddenly helpless and very sick. 5/17: Died. Autopsy: Much enlarged liver; spleen discolored. Formed feces in digestive tract. Heart distended with blood.

*Basal diet without cod-liver oil or calcium:*

No. 264, male, Group VIII. 5/22: Segregated because of stumbling in straw. Droopy but able to eat by itself. A new basal diet given, same as original Diet 11, except that cod-liver oil and $CaCO_3$ were omitted. 5/25: Now helpless, must be fed by hand but eats well. 5/28: Found dead. Autopsy: Eyes shrunken, with much whitish exudate. Eyeballs soft. Joints stiffened but not immobilized. Mottled liver; spleen discolored. Lungs normal. Auricles and right ventricle distended with blood.

*Basal diet without cod-liver oil and with a large amount of yellow corn meal:*

No. 250, female, Group VII. 5/22: Segregated because of stumbling in straw. Given the following basal diet: baked milk powder, 20 per cent; ground oats, 5 per cent; ground barley, 5 per cent; bran, 10 per cent; yellow corn meal, 48 per cent; yeast, 5 per cent; NaCl, 1 per cent; $CaCO_3$, 1.5 per cent; ferric ammonium citrate, 0.5 per cent; alfalfa meal, 4 per cent. 5/27: Now entirely helpless, must be fed by hand but eats well. 5/29: Found dead. Autopsy: Legs very stiff. Eyes watery; eyeballs soft. Liver pale and mottled, discolored around bile ducts. Spleen shrunken and brownish.

*Addition of dried alfalfa ad lib.:*

No. 303, male, Group VIII. 5/11: Segregated because of stumbling in straw. Was able to eat independently. Given all the dried alfalfa leaves it could eat in addition to the basal diet. 5/22: Still in good shape; eats well. To insure enough alfalfa, one feeding a day of alfalfa meal was made into a mash with water and was given by hand. 5/25: Entirely helpless but eats well. 6/2: Very weak; eyes contain exudate; hard to feed. 6/3: Found dead. Autopsy: Eyes suffused with exudate; eyeballs soft. Exudate from nose. Body very stiff; adductors strongly contracted. Liver abnormal; spleen contracted. Lungs hemorrhagic.

*Addition of raw milk:*

No. 237, male, Group VIII. 5/11: Segregated because of stumbling in straw. Given 30 cc. raw milk morning and evening, in addition to the ordinary basal diet. 5/17: Helpless; eats fairly well. 5/24: Very weak; death imminent; killed. Autopsy: Abnormal liver and white infarcts in kidney.

*Addition of fresh carrot:*

No. 269, female, Group VII. 5/25: Segregated because of stumbling in straw. Given 20 g. fresh, grated carrot daily. Ate it very well. 6/1: Can now run about easily. Scratch reflex elicited but with difficulty. Righting reaction slow. 7/9: Weight continued to drop until 6/11; since then has gone up steadily. Animal appears entirely cured. Killed for autopsy. Autopsy: Eyeballs soft. Heart flabby, decidedly softer than normal. Yellowish gray areas in liver. Spleen and kidneys normal.

*Addition of dehydrated carrot:*

No. 238, female, Group VII. 6/27: Segregated because of stumbling in straw. Given 5 g. dehydrated carrot daily, approximately equivalent to 20 g. raw carrot. Feeding continued until 7/14. No improvement, but animal retained the body weight it had before feeding of dry carrot began. Animal feeble and sensitive to tickling. Experiment discontinued.

*Quantitative experiment to determine minimum protective level of fresh alfalfa:*

No. 238, female, Group VII. 5/24: Segregated because of stumbling in straw. Given 2 g. fresh alfalfa tips daily. 6/11: Scratch reflex easily elicited. Righting reaction slow. 6/27: Condition generally worse. Two grams fresh alfalfa daily fails to maintain healthy condition in slightly diseased guinea pig.

No. 266, female, Group VII. 5/24: Segregated because of stumbling in straw. Extremely strong scratch reflex. Given 5 g. fresh alfalfa tips daily. 6/11: Scratch reflex elicited, but not so easily. Righting reaction slow; stumbles in straw. 7/11: Entirely recovered from stumbling. Very thin, and appearance not good. No scratch reflex. Killed for autopsy. Autopsy: Eyeballs soft. Liver has the characteristic smooth appearance, the hepatic lobules being indistinct.

No. 251, female, Group VII. 5/24: Segregated because of stumbling in straw. Given 10 g. fresh alfalfa tips daily. 6/11: Scratch reflex elicited with difficulty. Righting reaction normal. Eyes a little watery. 7/11: Weight gain rapid. Apparently entirely cured. Killed for autopsy. Autopsy: Eyeballs soft. Ventricular tissue softer than in control heart. Liver has smooth appearance.

No. 265, female, Group VII. 5/24: Segregated because of stumbling in straw. Give 15 g. fresh alfalfa tips daily. 6/5: Runs well in straw. Righting reaction almost normal in speed. No scratch reflex. 6/8: Dismissed as cured.

*Summary of protocols.*—No modification in the ultimate result was made by the substitution of Basal Diet 11 for Basal Diet 10. A continuation of the basal diet and orange juice led to the development of the symptoms already described and to death. If fresh alfalfa in liberal amounts was added to the diet of lightly diseased animals, the animals quickly regained apparent health. This indicates that factor *pl* is present in abundance in fresh alfalfa. A quantitative experiment showed that 10 g. and 15 g. of alfalfa tips a day were restorative, but 5 g. per day was only partially successful, and 2 g. per day allowed the animals to decline. The small amount of alfalfa necessary for protection indicates the vitamin-like character of factor *pl*. Dried alfalfa given a slightly affected animal prolonged its life for perhaps 2 weeks, but death finally followed the development of the usual syndrome. The drying process therefore decidedly reduced factor *pl* in the alfalfa.

Raw carrot, 20 g. per day, given a lightly affected animal, caused slow but certain improvement. After about 6 weeks the animal appeared cured, but at autopsy it showed signs of residual disease. Dehydrated carrot, 5 g. per day, given another sick animal, was unable to effect any improvement, although during the 2½ weeks when it was being administered the animal was able to maintain its body weight. The indication is, therefore, that raw carrot contains factor *pl* but in much lower concentration than alfalfa, and also that drying greatly diminishes the amount of the factor in carrot.

The addition of extra calcium, viosterol, or cod-liver oil to the diet of lightly diseased animals caused no improvement. The animals sickened and died with the characteristic symptoms. Likewise the omission of cod-liver oil, viosterol, and calcium did not delay or modify the course of the disease after it had once become established. Replacing the cod-liver oil in the basal diet by large amounts of yellow corn meal was without remedial effect. Raw milk also was not remedial in the amount used. If factor *pl* is present in these products, it must be small in amount.

*Cooked green feed.*—Group X of the second series of experiments was given Basal Diet 11 and orange juice, and in addition 20 g. cooked kale per animal per day. This was changed to 40 g. cooked alfalfa after 3 months' feeding, because of the closing kale season. The green feed was left in 2 per cent NaCl overnight and was then cooked in steam for an hour. Table IV shows that at 15 weeks Group X had gained weight about equally with the animals of Group IX, which received a supplement of fresh green feed. This was true also after the experiment had continued 6 months. Externally the animals appeared to be in excellent condition and could not be distinguished from the controls. Nevertheless, autopsy showed definite signs of latent disturbance due to lack of factor *pl*. Of the 8 animals examined, 6 showed either the characteristic smooth appearance of the liver or flabby ventricular tissue or both. Eye changes were also found, which will be discussed in the next section. The controls from Group IX, killed at the same time, all had normal livers, hearts, and eyes.

Two guinea pigs suffering from factor *pl* deficiency to the extent that they could no longer walk in straw without stumbling were

given daily, in addition to their ordinary Basal Diet 11 and orange juice, a supplement of 15 g. alfalfa tips cooked as follows: The tips were put into boiling water in a closed container and were cooked 20 minutes. The feeding was continued for 22 days. The animals improved on the treatment and were able to run about, but they were very thin and their weight showed only slight increase. At the end of the experiment, the scratch reflex could still be easily elicited and the animals did not appear vigorous. They were autopsied, and both showed swollen pale livers in addition to eye changes.

Thus factor *pl* shows some resistance to careful cooking but is nevertheless considerably reduced in the process. Even 40 g. cooked alfalfa per animal per day was not sufficient to safeguard the tissues of healthy animals from the onset of early symptoms, and animals sufficiently advanced in the disease to show outward signs were helped only a little by the addition of 15 g. cooked alfalfa to their diet.

*Eye involvement following lack of factor pl.*—It was observed that animals suffering from deficiency of factor *pl* were apt to have flattened, almond-shaped eyes instead of the very round, bulging eye of the normal guinea pig. Animals succumbing to the disease exhibited eyeballs which were quite soft and sunken. The eyes were usually suffused with a thin watery and milky exudate. When removed, they were found to have become more spherical than the typical eye, through the disappearance of the corneal bulge and the equalization of the different diameters of the eye.

A comparison was made of the eyes of animals in Group X (supplement, 40 g. cooked alfalfa), with those of Group IX (supplement, 40 g. raw alfalfa). The animals were killed by ether, and the eyes removed from the orbit. After testing the softness of the eyeball, it was opened and the vitreous body carefully removed. This was placed on a glass plate and immediately compared with that of an animal from Group IX. Of the 8 animals from Group X thus examined, 7 were found to have vitreous humors definitely less gel-like than those from Group IX. Those of Group X exuded more liquid and held their shape less well than those of Group IX. Thus, eye differences between the animals of Groups IX and X were found, even though there was no difference in body weight, or, as far as could be seen, in general well-

being. However, at autopsy, the animals of Group X gave definite signs of liver and heart involvement.

Other examples of eye involvement follow. Animal No. 283, which had received 5 g. dehydrated carrot supplement and had become very stiff in the wrists, was examined shortly after death. The eyeballs were so soft that it was difficult to remove the musculature without cutting into them, and the vitreous bodies were very liquid.

Two animals, Nos. 282 and 242, which had received 15 g. cooked alfalfa daily from the time they first failed to get the hind legs through the bedding straw, were examined for eye condition. These animals were progressing very well, could right themselves, although more slowly than normally, and could get the hind legs through the straw. At autopsy, the vitreous humors of both were found to be more liquid and less firm than those of two control animals.

### THIRD SERIES OF EXPERIMENTS

In the third series of experiments the effort was made to approximate a diet of natural foods by the elimination of the milk powder used in the previous series and the reduction of the cod-liver oil to 0.25 per cent. Dried alfalfa meal of the best quality to be obtained locally was used to guard against any possible deficiency of vitamin A resulting from the low level of cod-liver oil. The animals were divided into two groups. They were given Basal Diet 12 with supplements as follows: Group XI, 15 females, 6 cc. fresh orange juice per animal per day and 20 g. fresh alfalfa per animal per day. (The alfalfa was changed to kale during the winter season, but alfalfa was again given in the spring.) Group XII, 18 females, 10 cc. fresh orange juice per animal per day. The experiment continued for 38 weeks in this form. The average weight gain of Group XI was 141.8 per cent, and of Group XII was 114.7 per cent.

The hair of the animals in Group XI was thick and soft, while that of Group XII, receiving no green feed, was harsh and seemed thin. The muscles of the thigh were carefully palpated in all animals. With no exception the muscles of the animals receiving no green feed were harder and less bulky than those of the controls. Flexion at the carpal joint was tested to show the shortening of the extensor muscle group of the lower fore leg. The leg was held extended at

the elbow joint, and the attempt was made to flex the foot. This was easily accomplished in the animals which had received green feed; but in the animals which had had no green feed, a definite resistance was felt which kept the foot from being flexed at right angles to the lower leg. There was no exception to the development of this tension, but some individuals showed it more strongly than others. The righting reaction, which was practically instantaneous in the animals receiving green feed, was definitely slow in the other animals; and the scratch reflex, which seems never to be developed in normal animals, could be elicited in some of the animals which had no green feed.

## DISCUSSION

Diets of natural foods, apparently adequate in the ordinary food constituents and recognized vitamins, were given groups of guinea pigs. These diets did not contain fresh green feed. The earliest indication obtained by us that the guinea pigs, so fed, were abnormal was that planarian worms fed certain tissues from these guinea pigs grew more slowly and ultimately developed a deficiency disease with a characteristic syndrome. When green feed in the form of fresh kale was given the guinea pigs, the worms receiving their tissues remained normal (Wulzen and Bahrs, 1935; and Bahrs and Wulzen, 1936). The time required to develop tissues disease-producing for planarians was 1 month, but at this time the guinea pigs themselves were entirely normal in appearance. However, when the guinea pigs received these same diets over a period of about 4 months, they developed a disease with a characteristic syndrome which was always fatal.

Symptoms suggestive of those obtained by us have been cited by workers in experimental scurvy. Smith (1927) found that, when life was prolonged for several months in a scorbutic guinea pig by feeding inadequate amounts of vitamin C, complete disuse of the posterior portion of the animal ensued. Pain sensibility remained in the portion affected. These symptoms were obtained by us when guinea pigs were given an abundant supply of vitamin C but were deprived of factor *pl*, as represented by fresh kale and alfalfa. It may be pointed out that the ordinary diet low in vitamin C is low or lacking in factor *pl*. The external signs of deficiency in factor *pl* take a con-

siderable time for complete development on supplemented scurvy-producing diets. While they might not be conspicuous in an animal dying quickly of acute scurvy, they could become predominant in an animal kept for a long time from acute scurvy by administration of inadequate amounts of vitamin C. Thus an animal suffering from "chronic scurvy" might be expected to show a combination of symptoms, some due to inadequate amounts of vitamin C but some also referable to lack or insufficiency of factor *pl.*

Skeletal musculature degeneration has been found in scorbutic animals by numerous investigators, and it is also one of the leading outward signs of lack of factor *pl.* Dalldorf (1929) has emphasized this as occurring in his scorbutic animals. Rinehart, Conner, and Mettier (1934) also repeatedly observed degenerative changes in the skeletal muscles of their guinea pigs suffering from chronic scurvy. Meyer and McCormick (1928) using guinea pigs for the study of scurvy, have noted the highly nervous condition of their animals and the development of permanent locomotor disabilities. Our animals showed extreme degeneration of the skeletal muscles, and their strongly developed scratch reflex indicated their high degree of nervous excitability. Even our completely immobilized animals showed higher sensitivity to sound than did the controls.

Muscular degeneration, produced entirely apart from scurvy, has been carefully studied by Goettsch and Papperheimer (1931) and by Madsen, McCay, and Maynard (1935). Both sets of investigators used apparently well-constructed diets containing adequate amounts of the recognized vitamins, vitamin C being given as orange juice or tomato juice. Goettsch and Papperheimer describe as the essential and primary lesion produced in their guinea pigs a degeneration of the skeletal muscle which they consider to be the ultimate cause of death. The muscles were atrophied, pale, and less translucent than normal. Their findings correlate with ours; but by hand feeding we have been able to maintain the life of our animals for considerable periods of time after they had become completely immobilized, and we are not certain as to the underlying cause of death. Madsen, McCay, and Maynard in their recent memoir (1935) have summarized their extensive work on the toxicity of cod-liver oil for Herbivora. Madsen (1936) has further elaborated on their findings;

and Turner, Meigs, and Converse (1936) have also obtained muscular dystrophy by means of cod-liver oil. The prevailing muscular degeneration which they describe is so similar to that of our guinea pigs that we consider the same causes operative in each case.

Soft, flabby heart musculature is a characteristic of guinea pigs suffering from *pl* deficiency. The liver is inclined to hypertrophy and may show either abnormally distinct lobulation or may be unusually smooth in appearance and have indistinct hepatic lobules. The liver is commonly mottled and pale with indication of pronounced fatty degeneration. The spleen may be either contracted and discolored or hypertrophied. Meyer and McCormick (1928) note similar liver changes in their scorbutic animals, and they have found degenerative changes in the cardiac musculature. Madsen, McCay, and Maynard (1935) list the same sort of liver and heart abnormalities in the Herbivora with which they have experimented.

Goettsch and Pappenheimer (1931) consider that the lesions described by them must be referred to some still unknown factor, and make passing reference to a possible protective effect of green foodstuffs. They offer arguments to demonstrate that their animals were free from latent scurvy. These we corroborate. We found our animals at autopsy free from gross hemorrhages, and the teeth were always firmly set in the jaw. Moreover, we gave one of our lightly diseased animals daily doses of 0.005 g. ascorbic acid for a week and could see no amelioration of the symptoms. Had as little as 5 g. of fresh alfalfa been used instead of the ascorbic acid, there would have been decided improvement. Madsen, Maynard, and McCay (1935) also consider that scurvy is not a complicating factor in their results.

According to Madsen and his co-workers, both cod-liver oil and some other element of their synthetic diet are causative factors in the production of the lesions cited by them. They find that the complete omission of a cod-liver oil product from the synthetic diet does not entirely eliminate the injury, and that the addition of cod-liver oil to a diet of natural foods produces the characteristic muscle lesions in their animals.

With diets of natural foods we have succeeded in producing degenerative muscular changes in guinea pigs when the amount of cod-liver oil in the diet was as low as 0.25 per cent. In contrast to this

we have obtained optimum growth and development in guinea pigs fed as high as 4 per cent cod-liver oil for the period of the experiment, 9 months. The animals were in prime condition, fat and sleek. They never showed a trace of stiffness. Whether or not slight muscular lesions occurred at the beginning of the experimental period we cannot say; but the effect, if any, must have been exceedingly small and of no ultimate consequence. We have accomplished this result by the use of certain specific foods which appear to contain a necessary accessory factor, i.e., fresh kale, fresh alfalfa, and fresh carrots. Dried alfalfa, cooked alfalfa, and dried carrots also contain the factor, but in much smaller quanitities. It seems to be present in very slight amounts in yellow corn meal and milk, and may be considered almost or entirely lacking in orange and tomato juice. Turner, Meigs, and Converse (1936) have shown that in rabbits a good quality of alfalfa hay added to a diet which was producing muscular dystrophy enabled animals to survive, and that growth and reproduction appeared only partially impaired by cod-liver oil feeding.

Though these studies on the nutrition of the guinea pig are entirely an outgrowth of our findings in planarian nutrition, we are of the opinion that the factor with which we are dealing is the same as that with which Goettsch and Pappenheimer (1931) and Madsen, McCay, and Maynard (1935) are concerned. We also think it is possible that those investigators who have worked with chronic scurvy may have developed a complex of symptoms in their animals, one bloc of which is referable to lack of this same factor, often rendered more apparent by an excess of cod-liver oil in the diet.

## SUMMARY

1. A deficiency disease has been produced in planarian worms by feeding them tissues from guinea pigs given supposedly adequate diets in which vitamin C was furnished in the form of orange juice or tomato juice. When the guinea pigs received this diet over a period of several months, they also developed a deficiency disease, which invariably ended in death.

2. When the orange juice or tomato juice was replaced by a supplement of fresh alfalfa or kale, or these green feed supplements

were given in addition to the fruit juices, the guinea pigs remained in excellent condition throughout the experiment.

3. The gross morphology of the diseased animals showed extreme degeneration of the skeletal muscles, leading to immobilization of the entire body, degenerative changes in liver, heart, and kidneys, resorption of bone, and softened eyeballs.

4. The diseased animals differed from the controls in that they had lower body temperature, could not maintain their temperature as well in a cold environment, and showed higher nervous irritability.

5. Fresh kale and fresh alfalfa were found to contain an abundant supply of the active principle; fresh carrot, a moderate amount. Dehydrated alfalfa and carrot were found to have a low store of the active substance.

6. The modification of the basal diet to increase or eliminate cod-liver oil, viosterol, and calcium had no appreciable effect on the course of the disease already established. Neither did the addition of a plentiful supply of raw milk or a high proportion of yellow corn meal.

7. Carefully boiled alfalfa in liberal amounts retained enough of the active principle to maintain animals in an apparently healthy condition externally, but autopsy showed signs of disintegration of liver, heart, and eyeballs.

8. A basal diet containing 20 per cent dried alfalfa and 0.25 per cent cod-liver oil maintained the animals in normal condition for about a year, but the characteristic muscular dystrophy eventually developed.

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# STUDIES ON THE CARBOHYDRATE METABOLISM IN PLANARIANS

(Seven figures)

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IN VIEW of the predominant rôle of the carbohydrate metabolism both in free-living worms (for example, *Lumbricus*, *Tubifex*) and parasitic ones (*Ascaris*, *Fasciola*, *Moniezia*), it seemed worth while to investigate the matter also in free-living flatworms. Because they were easily obtainable, I have chosen for this purpose two planarians, *Planaria torva* and *Dendrocoelum lacteum*. As far as I know, there have never been published quantitative observations along this line, although Gelei (1912) and Prenant (1922) have shown with morphological methods that the planarians are able to store glycogen. On the other hand, Hyman, Willier, and Rifenburgh (1924) doubt whether any carbohydrate is to be found in these animals. These contradictions may be due to the different species used by the different investigators.

## MATERIAL AND METHODS

Most of the experiments were performed with *Planaria torva*, which was easy to find during the whole year in two small ponds near the laboratory. *Dendrocoelum lacteum* was collected in the same localities but was not obtainable as regularly and in the same quantity. Only fresh-collected animals were used. During the experiments they were kept in glass dishes in tap water without receiving food, and they were kept in the dark.

As everyone knows who has concerned himself with quantitative questions in planarians, it is difficult to weigh these worms accurately. I isolated them first with a hair pencil in a small, dry watch glass; removed, as far as possible, the last traces of water; and transferred the worms into a weighing glass, which I weighed rapidly with an accuracy of 1 mg. Then the worms were transferred

into the water of the experimental vessel, and the empty weighing glass was weighed again.

With a view to the different experimental objectives, the determinations of the carbohydrate content were made at different intervals. For each determination *Planaria torva* was used in groups of fifteen to forty animals (about 0.1–0.3 g.) and *Dendrocoelum lacteum* in groups of three to five animals (about 0.2 g.). Most of the carbohydrate determinations were made according to Simonovits' (1933) micro-modification of Pflüger's glycogen method, excepting that the sugar formed was determined according to Hagedorn and Jensen's method. In some experiments I used the method of Dische and Popper (1926), for total carbohydrate. In certain experiments the oxygen consumption was determined by means of a micro-modification of the Winkler method in general use in this laboratory. In others I used the new, more accurate oxygen titration described by Krogh (1935).

### CARBOHYDRATE CONTENT OF *Planaria torva* IN DIFFERENT SEASONS

As the observations concerning the polysaccharide content of *Planaria torva* were made fairly regularly from February, 1935, until November, 1935, it is possible to relate the storage of polysaccharide to the seasons. Figure 1 represents the data concerning this question. They demonstrate clearly that *P. torva*, like various other animals (mussels and snails, for example), shows a regular seasonal cycle in the polysaccharide content. In the autumn the polysaccharide content becomes very high, with a mean value of about 3 per cent of the fresh substance. This corresponds approximately to 15 per cent of the dry substance (dry substance, about 20 per cent; cf. Buchanan, 1931). During the winter and early spring the polysaccharide content diminishes slowly, to about 1.4 per cent of the fresh substance at the end of April. It seems very probable that the storage of polysaccharide, which occurs in the autumn before the water becomes very cold, is biologically significant in providing the worms with a reserve of food substance on which they can live during the winter and spring until more food becomes available. It seems reasonable to assume that the planarians are less

active during the winter and that they will therefore not be as able to provide themselves with food, as they do during the warmer seasons. In the first days of May the polysaccharide content drops extremely rapidly, to the low value of about 0.5 per cent of the fresh substance; and it remains at this level during several weeks. This behavior is not easy to understand. My first thought, that it might be related to the sexual cycle, providing the eggs with a reserve of

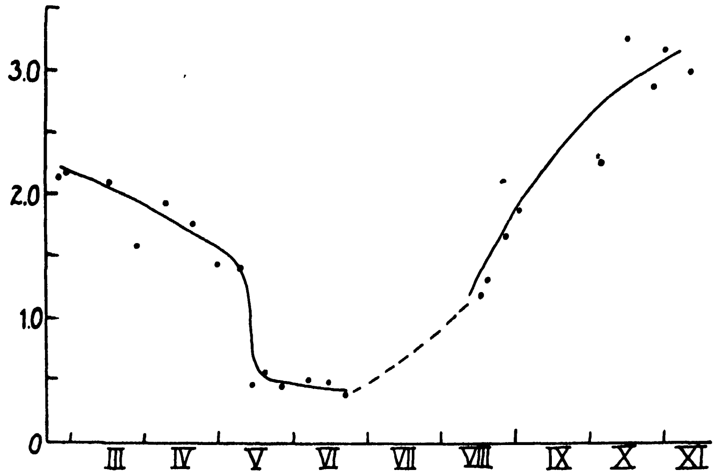


FIG. 1.—Variations of the polysaccharide content of *Planaria torva* at different seasons. Ordinates: polysaccharide content in percentage of the fresh substance; abscissas: months.

food, was not correct. An observation of the animal shows that they breed mainly during the months of May and June, when the polysaccharide content has already reached its minimum. The only relation between sexual life and polysaccharide content seems to be that the storage begins only when the breeding period has ended, an occurence which I found also in *Helix*. It would be interesting to investigate whether the changes in polysaccharide content are due principally or entirely to external factors, or, what seems more probable, also to internal factors. An investigation on animals regularly fed and kept at constant temperature would perhaps throw some light on this question.

## CARBOHYDRATE CONSUMPTION DURING STARVATION

The investigations of Child (1919) and Hyman (1919, 1920) have shown that the oxygen consumption and the carbon dioxide output of planarians, which can be taken as a measure of the total metabolism, decrease during the first days of starvation, but that they increase later, even above the level of fed animals, when the oxygen consumption is referred to the weight of the animals on the day of the determination. In my experiments it was only possible to deal with the first phase, as after a starvation of about a week the carbohydrate content was so low that a further continuation seemed not to be advisable, since less than about 1 mg. is difficult to determine with sufficient accuracy.

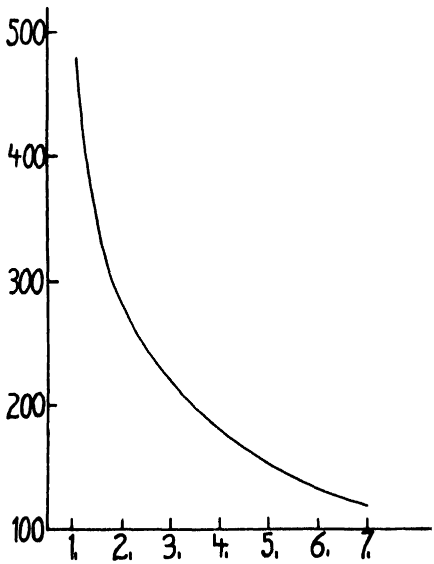


FIG. 2.—Rate of carbohydrate consumption in *Planaria torva* during the first week of starvation. Ordinates: milligrams of carbohydrate consumed by 100 g. of *Planaria torva;* abscissas: days of starvation.

The experiments have been performed at 25° C. Determinations of the polysaccharide content have been made at the beginning of the experiments and on the second, fourth, and seventh day of starvation. The data obtained in this way give the total amount of carbohydrate which has disappeared from the body on each respective day, compared with amount at the beginning of the experiment and referred to the initial weight of the worms. It is easy to deduce from this curve (Fig. 3) the curve of the daily polysaccharide consumption. The curve obtained in this way for *Planaria torva* is reproduced in Figure 2. It is similar to that of the above-mentioned authors for the total metabolism. The intensity of the carbohydrate consumption decreases during the first week of starvation. The rate

of decrease is greater during the first days, but diminishes later. During the first day, 100 g. of worms consume 0.48 g. of polysaccharide; by the seventh day, only 0.12 g. in 24 hours.

In addition to the experiments already mentioned, which were performed by the glycogen method, I have made a similar series of determinations by the method of Dische and Popper (1926) and obtained approximately the same curve. This shows clearly that during the first week of starvation polysaccharides are almost exclusively utilized. Otherwise the determinations by the method of Dische and Popper would have shown a greater decrease in the carbohydrate content.

It must be pointed out that these experiments were performed during February and March, when the worms had stored relatively large amounts of polysaccharide. Similar experiments performed during the summer months with planarians having only a small amount of polysaccharide demonstrate that in this case much less polysaccharide is consumed. I have carried out two experiments of this kind in June with worms containing, at the beginning, 0.75 per cent polysaccharide of the fresh substance. One hundred grams of worms consumed during 3 days, at 25° C., 0.43 g. of polysaccharide. The winter worms had consumed, during the same period and at the same temperature, 0.96 g. of polysaccharide. In comparative work on the carbohydrate metabolism of planarians it will therefore be necessary always to consider the seasons.

### THE RELATION BETWEEN TEMPERATURE AND CARBOHYDRATE METABOLISM

The intensity of the carbohydrate metabolism both in *Planaria torva* and *Dendrocoelum lacteum* is, in a high degree, dependent upon the temperature. I investigated the consumption of polysaccharide in *Planaria torva* and that of total carbohydrate in *Dendrocoelum lacteum*. Three different temperatures were used: 25°, 14°, and 2.5° C., which were kept constant during the experiments within a range of ±1° C. The results are summarized in Figures 3 and 4. They are drawn to the same scale and show that the intensity of the carbohydrate metabolism is much higher in *Planaria torva* than in *Dendrocoelum lacteum*. During the first four days of starvation

the consumption (in milligrams of carbohydrate per 100 g. of worms) amounted to:

| | Temperature (Centigrade) | | |
|---|---|---|---|
| | 25° | 14° | 2.5° |
| *Planaria torva* | 1,100 | 480 | 260 |
| *Dendrocoelum lacteum* | 360 | 150 | 60 |

All these experiments were carried out during the winter and early spring. At this time the polysaccharide stored by *Planaria torva* was approximately 2 per cent of the fresh substance, the total

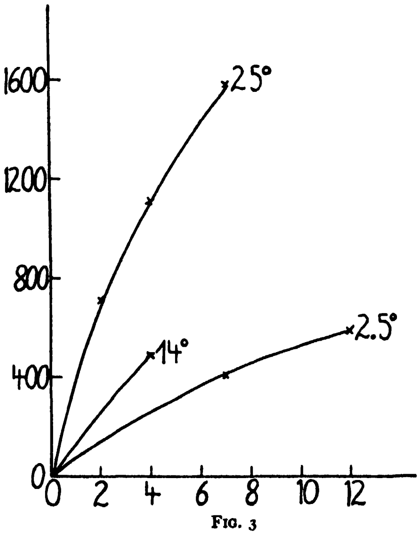


Fig. 3

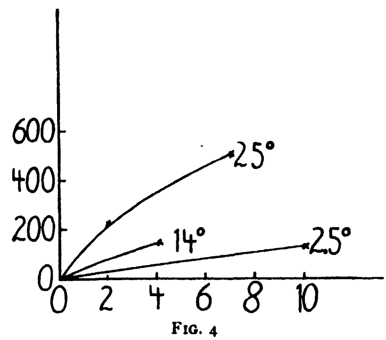


Fig. 4

Fig. 3.—Carbohydrate consumption in *Planaria torva* at different temperatures. Ordinates: milligrams of carbohydrate consumed by 100 g. of *Planaria torva;* abscissas: days of starvation. Temperature in degrees Centigrade.

Fig. 4.—Carbohydrate consumption in *Dendrocoelum lacteum* at different temperatures. Co-ordinates as in Figure 3.

carbohydrate by the method of Dische and Popper (1926) being approximately 2.5 per cent. *Dendrocoelum lacteum* had only 1 per cent of the total carbohydrate in the fresh body, of which only half

of the carbohydrates present at this time were polysaccharides determinable by Pflüger's method. Concerning the above-mentioned difference between the utilization of carbohydrate in winter and summer in *Planaria torva*, it seems reasonable to assume that in this case also the difference in the quantity of carbohydrates stored in the two species may explain the variation in the intensity of carbohydrate metabolism which is apparent at all temperatures used.

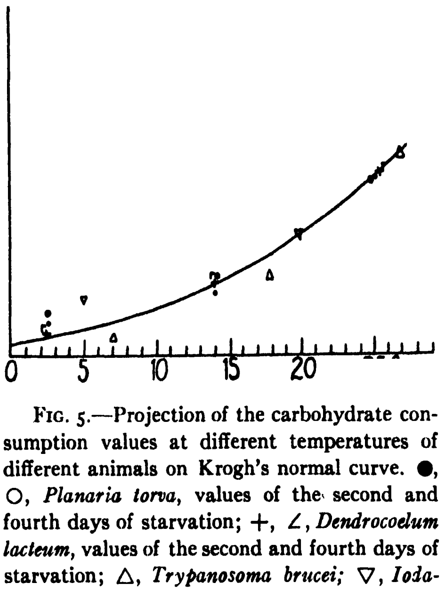


FIG. 5.—Projection of the carbohydrate consumption values at different temperatures of different animals on Krogh's normal curve. ●, ○, *Planaria torva*, values of the second and fourth days of starvation; +, ∠, *Dendrocoelum lacteum*, values of the second and fourth days of starvation; △, *Trypanosoma brucei;* ▽, *Iodamoeba bütschlii.*

A closer study of Figures 3 and 4 demonstrates that the increase in intensity in the carbohydrate metabolism with rising temperature does not conform to the rule of van't Hoff. I (1933) had formerly found the same in the case of the sugar metabolism of *Trypanosoma brucei*. The curve obtained at that time was more like the normal curve published by Krogh (1914) for the oxygen consumption of several species of animals. On the suggestion of Professor Krogh I have now projected on the normal curve the figures for the carbohydrate consumption of the first two and the first four days of starvation, as obtained from Figures 3 and 4, and have done the same with the previous data for the sugar metabolism of *T. brucei* and the glycogen consumption in the cyst of *Iodamoeba bütschlii*. The result of this procedure is shown in Figure 5. It is probable, but by no means certain, that Krogh's normal curve adequately expresses the relation between temperature and carbohydrate metabolism. At very low temperature the carbohydrate metabolism of these animals, and especially of the planarians, seems to be higher than the curve would indicate; but the difference may be due to experimental errors. This kind of experiment is more difficult

to perform in the case of carbohydrate metabolism than in that of oxygen consumption. In the latter it is possible to work on the same animal or the same group of animals at the different temperatures. Such a procedure is impossible in studying the carbohydrate metabolism, or at least in the species used in my experiments. It is obvious that the use of different animals must increase the error.

I have studied in four series of experiment—two on *Planaria torva* and two on *Dendrocoelum lacteum*—the relation of oxygen consumption to temperature in the same range as in the carbohydrate experiments, using also in this case three different temperatures in each series. The values obtained have again been projected on the normal curve (Fig. 6). We find here an irregular deviation from the curve, which indicates a better agreement than in the case of the carbohydrate metabolism. It seems very probable that Krogh's normal curve gives the best approximation for the relation of oxygen consumption to temperature in planarians also.

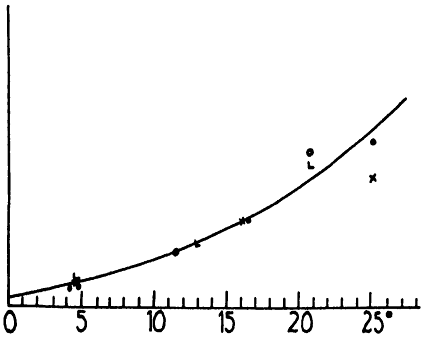


FIG. 6.—Projection of the oxygen consumption values at different temperatures of *Planaria torva* and *Dendrocoelum lacteum* on Krogh's normal curve. ●, ○, *Planaria torva*, first and second series; ×, ∠, *Dendrocoelum lacteum*, first and second series.

## CARBOHYDRATE METABOLISM AND TOTAL METABOLISM

Several species of worms obtain the energy required for their vital processes mainly from carbohydrates. This is true, in the first place, of different parasites, as shown by investigations on the metabolism of *Ascaris* (Weinland, 1901; Schulte, 1917; von Brand, 1934; and others), *Fasciola* (Weinland and von Brand, 1926), and *Moniezia* (von Brand, 1933). The rate of consumption of carbohydrates is very high in these cases, as these organisms are not able to obtain appreciable amounts of free oxygen under biological conditions. Their type of metabolism is mainly a fermentation. From this pro-

cess much less energy can be derived than would be obtained from a total oxidation of the sugar, and the necessary consequence is a very intense rate of carbohydrate metabolism. But we know also, in free living worms, cases with high rates of carbohydrate consumption. Lesser (1908) has shown that the earthworm uses, at least in the first days of starvation, glycogen almost exclusively, with some protein. Much glycogen is also used by the polychaete worms *Owenia*, *Halla*, and *Spirographis* (von Brand, 1927). On the other hand, we know that the leech lives mainly on protein (Pütter, 1907, 1908; Lafargue and Fayemendy, 1932). These differences made it desirable to investigate the part which carbohydrate metabolism plays in the total metabolism of planarians. We have two ways to solve such a question: It is possible to determine the respiratory quotient and to draw conclusions from it concerning the level of sugar consumption in comparison with the total metabolism. I have made no determinations of this kind in planarians. I chose the second possible way: As mentioned above, direct analyses of the carbohydrate consumption were made. Furthermore, I have determined the oxygen consumption at 25° C. in two series each on *Planaria torva* and *Dendrocoelum lacteum*. These experiments extended over a period of 1 week, determinations being carried out in single periods of 24–48 hours. For both species a curve results which is representative of the total metabolism. Since we know the rate of carbohydrate consumption, it is possible to calculate the amount

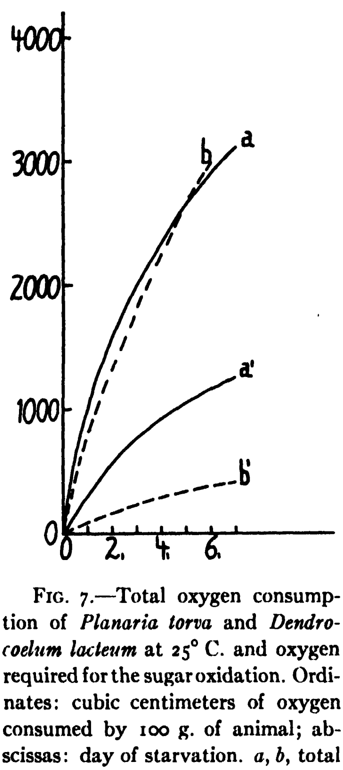


FIG. 7.—Total oxygen consumption of *Planaria torva* and *Dendrocoelum lacteum* at 25° C. and oxygen required for the sugar oxidation. Ordinates: cubic centimeters of oxygen consumed by 100 g. of animal; abscissas: day of starvation. *a*, *b*, total oxygen consumed by 100 g. of *Planaria torva:* (*a*) by 100 g. of *Dendrocoelum lacteum;* (*b*) *a′*, *b′*, oxygen required for the oxidation of sugar by 100 g. of *Planaria torva* (*a′*); by 100 g. of *Dendrocoelum lacteum* (*b′*).

of oxygen required for the oxidation of the sugar. We have to assume that the sugar is completely oxidized. This is not strictly proved, but is very probable, as Child (1919) has shown that the only acid produced by planarians is carbon dioxide. From our knowledge of the metabolism of other worms it is reasonable to assume that an incomplete oxidation of sugar in planarians would also lead to the formation of other acids.

Figure 7 shows the curves both for *Planaria torva* and *Dendrocoelum lacteum*. We can draw from them mainly two conclusions: First, the total rate of oxygen consumption of the two species is similar. It is relatively high but is in approximate accordance with those published by other authors (Hyman, 1919, 1920; and Allen, 1919) for other species of planarians. Second, the curves demonstrate clearly—which is somewhat amazing—that the proportion between total oxygen consumption and oxygen required for the oxidation of the carbohydrates remains practically the same during the single days of the first week of starvation. The proportion is, in the case of *Planaria torva*, about 1 to 0.3–0.4; in that of *Dendrocoelum lacteum*, according to the lower rate of carbohydrate metabolism, only 1 to 0.12–0.15. These figures demonstrate that *Planaria torva* uses during the first week of starvation about one-third, and *Dendrocoelum lacteum* about one-eighth, of the total oxygen in the carbohydrate metabolism. It is to be expected that in later stages of starvation the relative rate of carbohydrate metabolism will be reduced because the stores are almost exhausted after one week at 25° C.


## SUMMARY

1. *Planaria torva* shows cyclical variations in the polysaccharide content.

2. The rate of carbohydrate metabolism decreases in *P. torva* during the single days of the first week of starvation at 25° C.

3. The rate of carbohydrate metabolism is higher in *P. torva* during the winter, when the worms have stored much polysaccharide, than during the summer, when the amount of polysaccharide stored is small.

4. The intensity of carbohydrate metabolism both in *P. torva* and *Dendrocoelum lacteum* is dependent, in high degree, upon the tem-

perature. The curve obtained for its increase with rising temperature does not conform to the van't Hoff curve but is much more like Krogh's normal curve.

5. Krogh's normal curve is an adequate expression for the relation of oxygen consumption to temperature in planarians.

6. *Planaria torva* uses during the winter about one-third of the total oxygen consumed in the carbohydrate metabolism; *Dendrocoelum lacteum*, only one-eighth.

7. In both species the proportion of carbohydrate metabolism to total metabolism remains the same during the single days of the first week of starvation.

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